

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
10 May 2001 (10.05.2001)

PCT

(10) International Publication Number  
WO 01/32009 A1

- (51) International Patent Classification<sup>7</sup>: A01K 67/027, 67/033, A01N 63/00, 65/00, C12N 15/00, 15/09, 15/63
- (21) International Application Number: PCT/US00/05713
- (22) International Filing Date: 3 March 2000 (03.03.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
09/431,901 2 November 1999 (02.11.1999) US
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:  
US 09/431,901 (CIP)  
Filed on 2 November 1999 (02.11.1999)
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- (81) Designated States (*national*): AU, CA, JP, US.
- (84) Designated States (*regional*): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
- Published:  
— With international search report.
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*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 01/32009 A1

(54) Title: PROPAGATION OF HUMAN HEPATOCYTES IN NON-HUMAN ANIMALS

(57) Abstract: The present invention relates to the preparation of non-human animals having chimeric livers, whereby some or substantially all of the hepatocytes present are human hepatocytes. It is based, at least in part, on the discovery that rats, tolerized *in utero* against human hepatocytes, were found to serve as long-term hosts for human hepatocytes introduced post-natally, and the introduced hepatocytes maintained their differentiated phenotype, as evidenced by continued production of human albumin. The present invention further relates to the use of such animals as models of various liver diseases, including viral infection. Such embodiments are based on the discovery that transplanted human hepatocytes in chimeric livers were found to be susceptible to HBV infection *in situ* and supported replication of the virus as evidenced by the appearance of HBsAg in serum and in liver cells and by the presence of covalently closed circular DNA forms in liver.

## PROPAGATION OF HUMAN HEPATOCYTES IN NON-HUMAN ANIMALS

### INTRODUCTION

The present invention relates to the propagation of human hepatocytes in the livers of non-human animals that have been tolerized to the human cells. Such animals provide an in vivo model system of the human liver that may be used in toxicology assays and in the study of human liver diseases, including the various forms of hepatitis (in particular hepatitis B and C) and alcohol - induced liver degeneration. They may also be used as a source of human hepatocytes for reconstitution of liver tissue, thereby providing an alternative to liver transplantation.

10

### 2. BACKGROUND OF THE INVENTION

#### 2.1. THE NEED FOR A CULTURE SYSTEM FOR HUMAN HEPATOCYTES

To accurately study the physiology of human liver cells (hepatocytes), scientists need a model system in which the hepatocytes exist as they would in the intact liver. Such systems have proven to be difficult to achieve, because when hepatocytes are removed from their native environment, they tend to lose their specialized functions, or "de-differentiate". The loss of liver-specific functions makes it difficult or impossible to study the normal functions of hepatocytes as well as their response to chemical or biological agents. For example, research directed toward infectious diseases of the liver, in particular viral hepatitis, has been hampered by the lack of an adequate model system. Hepatitis B and hepatitis C, and the problems that have been encountered by scientists studying these infectious and dangerous viruses, are discussed in the following subsections.

In addition, a system for propagating human hepatocytes could be used to provide cells that could be used as an alternative or adjunct to liver transplant. Currently, patients suffering from liver disease may have to wait for long periods of time before a suitable organ for transplant becomes available. After transplant, patients need to be treated with immunosuppressive agents for the duration of their lives in order to avoid rejection of the donor's liver. A method for propagating the

patient's own cells could provide a source of functional liver tissue which would not require immunosuppression to remain viable.

## 2.2 HEPATITIS B VIRUS

Hepatitis B virus ("HBV") is the prototype of the *Hepadnaviridae*, characterized by a unique genome structure comprising partially double-stranded DNA ( *Fields Virology*, 1996, Third Edition, Fields, et al. eds., Lippincott-Raven, New York, pp. 2741-2742). In the United States, there are about a million carriers of HBV, and the number of carriers in the world exceeds 350 million ( *Fields Virology*, p. 2741; Petersen et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:310-315). In addition to causing an acute hepatitis, viral infection may lead to chronic infection and consequent liver failure and/or the development of hepatocellular carcinoma ( *Fields Virology*, pp. 2748-2751).

One of the major problems in the study of human viral hepatitis is the lack of convenient laboratory models of the disease. The narrow host specificity of the viruses limits current infection models to high primates (Caselmann, 1994, Antiviral Res. 24:121-129). Although HBV expressing immortalized human hepatocytes as isolated cells (Brown et al., 2000, Hepatology 31:173-181) or human liver fragments previously infected with hepatitis B virus (HBV) have been transplanted into immunodeficient rodent hosts (Ilan et al., 1999, Hepatology 29:553-562), and have been shown to continue to produce virus and antigens, it has not been shown whether normal human hepatocytes can remain sufficiently differentiated in a foreign host to be susceptible to infection by HBV. Among the murine models recently developed are a transgenic mouse model and a "Trimera", reported in Petersen et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:310-315 and Ilan et al., 1999, Hepatology 29:553-562, respectively.

In the transgenic mouse model of Petersen et al., a transgene encoding a hepatotoxic urokinase-type plasminogen activator was introduced into RAG-2 knockout mice, which lack mature B and T lymphocytes, and then woodchuck hepatocytes were introduced via splenic injection. The woodchuck hepatocytes replaced up to 90 percent of the mouse liver, and supported woodchuck hepatitis virus

(another hepadnavirus) replication indefinitely. The replication of the virus responded to pharmacologic agents.

In the Trimera model described by Ilan et al., normal mice were preconditioned by lethal total body radiation, radioprotected with SCID mouse bone marrow cells, and then engrafted with human liver fragments infected *ex vivo* with hepatitis B.

### 2.3. HEPATITIS C VIRUS

Hepatitis C virus was first characterized in 1989 (Choo et al., 1989, Science 244: 359-362), but its existence had been posited for many years as an elusive entity that caused flu-like symptoms in certain patients who had received blood transfusions. Because these symptoms were sometimes followed, years later, by liver disease, the clinical syndrome was referred to as non A - non B hepatitis ("NANBH").

Hepatitis C virus ("HCV") is now known to be a member of the *Flaviviridae* family of viruses, which includes viruses that cause bovine diarrhea, hog cholera, yellow fever, and tick-borne encephalitis (Kato et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87: 9524-9528; Choo et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88: 2451-2455; Okamoto et al., 1991, J. Gen. Virol. 72: 2697-2704; Takamizawa et al., 1991, J. Virol. 65: 1105-1113). The viral genome consists of an approximately 9.5kb single - stranded, positive - sense RNA molecule characterized by a unique open reading frame coding for a single polyprotein (reviewed in Clarke, 1997, J. Gen. Virol 78: 2397-2410 and Major and Feinstone, 1997, Hepatology 25: 1527-1538). Based upon phylogenetic analysis of the core, EI, and NS5 regions, HCV has been found to be genetically heterogeneous, with at least six genotypes and more than 30 subtypes dispersed throughout the world (Major and Feinstone, 1997, Hepatology 25: 1527-1538; Clarke, 1997, J. Genl. Virol 78: 2397-2410).

HCV has been estimated to infect 170 million people worldwide, which is more than four times the number of persons infected with human immunodeficiency virus ("HIV"), and the number of HCV-associated deaths may eventually overtake deaths caused by AIDS (Cohen, 1999, Science 285: 26-30). The Center for Disease Control has calculated that HCV may be harbored by 1.8 percent of



the U.S. population. (*Id.*). The only available therapy is interferon, but most HCV isolates are resistant (Thomas et al., 1999, *Hepatology* 29: 1333), although more promising results were obtained when interferon was combined with ribavirin (Cohen et al., 1999, *Science* 285: 26-30 citing Poynard et al., 1998, *Lancet* 352:1426-1432 and Davis et al., 1998, *N. Engl. J. Med.* 339:1493-1499). Unfortunately, the interferon/ribavirin combination is less effective against the most common HCV genotype found in the U.S., with only 28 percent of persons infected with that genotype exhibiting a sustained response to treatment (Davis et al., 1998, *N. Engl. J. Med.* 339:1493-1499).

10           The development of more successful forms of therapy (and our understanding of HCV biology) has been hampered by the absence of a good model system for HCV infection. Only humans and certain higher primates are susceptible to infection (Feinstone et al., 1981, *J. Infect. Dis.* 144: 588). A variety of mammalian cell systems which support the growth of HCV have been reported which rely on the use of strand-specific RT-PCR as evidence of virus replication (Major and Feinstone, 15           1997, *Hepatology* 25:1527-1538 citing Mitzutani et al., 1995, *Biochem. Biophys. Res. Commun.* 212: 906-911; Shimizu and Yoshikura, 1994, 68: 8406-8408; Kato et al., 1995, *Biochem. Biophys. Res. Commun.* 206: 863-869; Cribier et al., 1995, *J. Gen. Virol.* 76: 2485-2491; and Yoo et al., 1995, *J. Virol.* 69: 32-38).

20           As reviewed in Clarke (*supra*), there have been reports of viral replication in systems based on hepatic tissue (Ito, et al., 1996, *J. Gen. Virol.* 77: 1043-1054), peripheral blood mononuclear cells (Willems et al., 1996, *J. Med. Virol.* 42: 272-278; Zignego et al., 1992, *J. Hepatology* 15: 382-386), human T and B cell lines (Bertolini et al., 1993, *Res. Virol* 144: 281-285; Shimizu et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89: 5477-5481), human fetal liver cells (Iacovacci et al., 1993, *Res. Virol.* 144: 275-279), chimpanzee hepatocytes (Lanford et al., 1994, *Virol.* 202: 606-614), Daudi B-cells (Nakajima et al., 1996, *J. Virol.* 70: 3325-3329), and the human T cell leukemia virus type I-infected T cell line MT-Z (Mitzutani et al., 1995, *Biochem. Biophys. Res. Comm.* 212: 906-911; Sugiyama et al., 1997, *J. Gen. Virol.* 78: 329-336). None of these systems has, however, proved satisfactory.

Hepatitis C infected human liver tissue was transplanted into Trimera mice described in the preceding section, as reported by Galun et al., 1995, J. Infect. Dis. 172:25-30.

5 A newer system was recently reported by Lohmann et al. (1999, Science 285: 110-113) in which subgenomic HCV RNA replicons were transfected into a human hepatoma cell line and found to replicate to high levels. Nonetheless, this system does not generate virus and therefore is not a model of productive infection (Cohen, *supra*).

### 2.3. HEPATOCYTES FOR LIVER RECONSTITUTION

10 Reconstitution of liver tissue in a patient by the introduction of hepatocytes (also referred to as "hepatocyte transplantation") is a potential therapeutic option for patients with acute liver failure, either as a temporary treatment in anticipation of liver transplant or as a definitive treatment for patients with isolated metabolic deficiencies (Bumgardner et al., 1998, Transplantation 65: 53-61). Animal  
15 models have been developed for studying the effectiveness of hepatocyte transplantation in the context of pharmacologically or surgically induced liver failure (*Id*, citing Mito et al., 1993, Transplant Rev. 7: 35; Takeshita et al. 1993, Cell Transplant 2: 319; Sutherland et al., 1977, Surgery 82: 124; Sommer et al., 1979, Transplant Proc. 9: 578; and Demetriou et al., 1988, Hepatology 8: 1006), or for the  
20 treatment of isolated errors of metabolism (Wiederkehr et al., 1990, Transplant 50: 466; Onodera et al., 1995, Cell Transpl. 4 (Supp. 1): 541; Cobourn et al., 1987, Transpl. Proc. 19: 1002; Rozga et al., 1995, Cell Transplant 4: 237; Kay et al., 1994, Hepatology 20: 253; Matas et al., 1976, Science 192: 892; Holzman et al., 1993, Transplantation 55: 1213; Moscioni et al., 1989, Gastroenterol. 96: 1546; Groth et al.,  
25 1977, Transplant Proc. 9: 313). Use of transfected hepatocytes in gene therapy of a patient suffering from familial hypercholesterolemia has been reported in Grossman et al., 1994, Nat. Genet. 6: 335.

A major obstacle to achieving therapeutic liver reconstitution is immune rejection of transplanted hepatocytes by the host, a phenomenon referred to  
30 (where the host and donor cells are genetically and phenotypically different) as

"allograft rejection". Immunosuppressive agents have been only partially successful in preventing allograft rejection (Javregui et al., 1996, *Cell Transplantation* 5: 353-367, citing Darby et al., 1986, *Br. J. Exp. Pathol.* 67: 329-339; Maganto et al., 1988, *Eur. Surg. Res.* 20: 248-253; Makowka et al., 1986, *Transplantation* 42: 537-541).

- 5 The three main alternative approaches which have been explored are 1) physically shielding transplanted cells from the host immune system, for example, in an alginate - polylysine or chitosan capsule; 2) depletion of antigen presenting cells; or 3) induction of alloantigen - specific tolerance in the host (Javregui et al., *supra*). Chowdhury has tested the hypothesis that intrathymic injection of donor rat
- 10 splenocytes may result in suppression of allograft hepatocyte rejection in peripheral lymphocyte depleted adult rats (Jauregui et al., *supra*, citing Fabrega et al., 1995, *Transplantation* 59: 1362-1364). In that study long-term tolerization occurred with administration of splenocytes but not hepatocytes.

- For successful reconstitution, the age of the donor cells has been
- 15 considered significant. Cusick et al. (1997, *J. Ped. Surg.* 32: 357-360) report that transplanted fetal hepatocytes had a significant survival advantage over adult hepatocytes, independent of recipient age. However, Rhim et al. (1994, *Science* 263: 1149-1152) demonstrated that adult mouse liver cells could proliferate when introduced into the livers of congenic transgenic mice carrying a hepatotoxic
- 20 transgene (urokinase under the control of the albumin promoter, which is liver-specific and only active postnatally). The donor cells were observed to have divided at least 12 times (reconstitution of an entire liver from one hepatocyte would require 28 cell doublings). Other references relating to liver repopulation in immunologically deficient animals with transplanted hepatocytes include Gupta et al., 1996, *Prog. Liv*
- 25 *Dis.* 14:199-222; Sandgren et al., 1991, *Cell* 66:245-256; and Rhim et al., 1995, *Proc. Natl. Acad. Sci. U.S.A.* 92: 4942-4946.

### 3. SUMMARY OF THE INVENTION

The present invention relates to the preparation of tolerized non-human animals having chimeric livers, wherein some or a majority of the hepatocytes present are human hepatocytes. It is based, at least in part, on the discovery that rats, tolerized  
5 *in utero* against human hepatocytes, were found to serve as long-term hosts for human hepatocytes introduced postnatally, and that the introduced hepatocytes maintained their differentiated phenotype, as evidenced by continued production of human albumin.

In a first embodiment, the present invention provides for a method of  
10 preparing a non-human animal having a liver comprising human hepatocytes, comprising (i) inducing tolerance in an immunocompetent host non-human animal, where the animal is preferably a fetus or a neonate; and (ii) introducing human hepatocytes into the tolerized animal, preferably postnatally and preferably by intra-splenic injection. In specific non-limiting embodiments, the host animal is subjected  
15 to a selection pressure which favors survival and/or proliferation of human, rather than host animal, hepatocytes.

In a second embodiment of the invention, an animal having a chimeric liver, prepared as described above, may be used as a model system for human hepatocyte function in a toxicology study. Because the human hepatocytes maintain  
20 their differentiated state and are situated in their natural anatomic location, this model system recapitulates the metabolic fate of test agents as they pass from the site of administration through the liver.

In a third embodiment of the invention, an animal having a chimeric liver may be used as a model system for human liver disease. Such model systems are  
25 particularly useful for diseases which specifically effect human (or primate), but not non-human (or non-primate) livers, such as hepatitis B and hepatitis C infection and alcohol-induced liver degeneration/fibrosis. Immunocompetent chimeric animals of the invention exhibit the further advantage of having an immune system which is intact but for exhibiting tolerance toward the human cells comprised in the animal's  
30 liver.

In a fourth embodiment of the invention, an animal having a chimeric liver may be used as a source of human hepatocytes which may be used therapeutically. As non-limiting examples, such human hepatocytes may be used in gene therapy applications or to reconstitute liver tissue in a human host whose own liver has been substantially damaged. Large animals having a chimeric liver may be particularly desirable for such embodiments.

#### 4. DESCRIPTION OF THE FIGURES

FIGURE 1.  $^3\text{H}$ -thymidine incorporation in mixed lymphocyte assays where the responder cells were rat spleen cells and the stimulator cells were irradiated human hepatocytes. "Spleen (iu)" designates spleen cells from rats tolerized by transuterine injection of human hepatocyte lysates, and "spleen (iu/is)" designates spleen cells from rats tolerized as fetuses with human hepatocyte lysates followed by intrasplenic transplantation of human hepatocytes after birth.

FIGURE 2. Western blot, using anti-human albumin antibody as a probe, of human serum albumin (lane 1), serum from a tolerized rat six weeks after intrasplenic injection (lane 2), and sera from a tolerized rat, injected with human hepatocytes, 24 hours (lane 3), and eight days (lane 4) after a second injection of human hepatocytes. Lane 5 contains serum from a non-tolerized rat eight days after a second injection of human hepatocytes.

FIGURE 3. Western blot, using anti-human albumin antibody as a probe, of human serum albumin (lane 1), rat serum albumin (lane 2), and serum from a tolerized rat that had received an intrasplenic injection of human hepatocytes, one week (lane 3), two weeks (lane 4) and three weeks (lane 5) after injection with human hepatocytes.

FIGURE 4A-D. Immunofluorescence studies using anti-human albumin as primary antibody and fluorescent Texas red - coupled secondary antibody. (A) Anti-human albumin antibody binding to control (non-chimeric) rat liver; (B) anti-human albumin antibody binding to chimeric rat liver three weeks after injection with human hepatocytes; (C) same as B, without secondary antibody visualization; and (D) anti-human albumin antibody binding to the liver of a rat that had been

tolerized with a human hepatocyte lysate but did not receive subsequent injection of viable liver cells.

FIGURE 5. The same section of chimeric rat liver shown in FIGURE 4B to express human albumin, stained with hematoxylin and eosin to demonstrate normal histology.

FIGURE 6A-D. Immunofluorescence studies using primary and secondary antibodies as in FIGURE 4A-D, showing (A) a section of liver from a tolerized rat six weeks after intrasplenic injection with human hepatocytes, stained with both antibodies; (B) as in (A), but without secondary antibody staining; (C) as in (A), but with no antibody binding; and (D) a section of liver from a non-tolerized rat, six weeks after intrasplenic injection of human hepatocytes, stained with both antibodies.

FIGURE 7. Western blot, using anti-human albumin antibody as a probe, of human serum albumin (lane 1), rat serum (lane 2) and sera from a chimeric rat which had been tolerized by intrathymic injection of human hepatocytes, at various times after intrasplenic injection with human hepatocytes (lane 3 = 2 days, lane 4 = 2 weeks, lane 5 = 3 weeks, lane 6 = 5 weeks, lane 7 = 6 weeks).

FIGURE 8A-F. Immunofluorescence studies of liver sections from tolerized rats injected with human hepatocytes and inoculated with hepatitis B virus (HBV) at 1 week, 6 weeks, and 14 weeks following inoculation, stained with anti-albumin primary and Texas red conjugated secondary antibody (FIGURES 8A, 8C and 8E, for weeks 1, 6 and 14, respectively) or anti-hepatitis B surface antigen (HBsAg) antibody and FITC-conjugated secondary antibody (FIGURES 8B, 8D, and 8F for weeks 1, 6 and 14, respectively).

FIGURE 9A-H. Immunofluorescence studies of liver sections from rats that were either (i) tolerized, injected with human hepatocytes, and inoculated with HBV (CA2)(FIGURES 9A and 9B); (ii) tolerized and injected with human hepatocytes but not inoculated (CA3) (FIGURES 9C and 9D); (iii) tolerized and inoculated with HBV, without injection of human hepatocytes (CA5) (FIGURES 9E and 9F); or tolerized, injected with human hepatocytes and inoculated with HBV (CA2) but not reacted with primary anti-albumin or anti-HBsAg antibodies

(FIGURES 9G and 9H). Sections were stained with anti-albumin primary and Texas red conjugated secondary antibody (FIGURES 9A, 9C, 9E, and 9G) or anti-HBsAg antibody and FITC-conjugated secondary antibody (FIGURES 9B, 9D, 9F, and 9H).

FIGURE 10. Photograph of an ethidium bromide stained gel of products of RT-PCR of human albumin mRNA, the lanes containing the RT-PCR products resulting from experiments using, as template, RNA from : lane 2=rat; lane 3=human; lane 4= HepG2.2.15; lane 5= Rat CA1 (tolerized, injected with human hepatocytes, subsequently inoculated with HBV); lane 6= Rat CA2 (tolerized, injected with human hepatocytes, subsequently inoculated with HBV); lane 7= rat CA3 (tolerized and injected with human hepatocytes but not inoculated with HBV); lane 8= rat CA4 (tolerized and injected with human hepatocytes but not inoculated with HBV); lane 9= (tolerized and inoculated with HBV, without injection of human hepatocytes); lane 10= (tolerized and inoculated with HBV, without injection of human hepatocytes); lane 11=rat CA7 (treated with saline, negative control); and where lane 1= 1,000 bp ladder.

FIGURE 11A-B. Photograph of an ethidium bromide stained gel of products of RT-PCR of human albumin mRNA (FIGURE 11A) and HBV RNA (FIGURE 11B), the lanes containing the RT-PCR products resulting from experiments using, as template, RNA from : lane 2=rat; lane 3=human; lane 4= HepG22.2.15; lane 5= Rat CA2 (tolerized, injected with human hepatocytes, inoculated with HBV) 1 week post-inoculation; lane 6= Rat CA2 6 weeks post-inoculation; lane 7= rat CA2 14 weeks post-inoculation, where lane 1 = 1,000 bp ladder.

FIGURE 12. Photograph of an ethidium bromide stained gel of products of RT-PCR of human hepatitis B viral RNA, the lanes containing RT-PCR products resulting from experiments using, as template, RNA from: lane 2= rat; lane 3= human; lane 4= HepG22.2.15; lane 5= Rat CA1 (tolerized, injected with human hepatocytes and inoculated with HBV); lane 6= Rat CA2 (tolerized, injected with human hepatocytes, inoculated with HBV); lane 7= Rat CA3 (tolerized with human hepatocytes but not inoculated with HBV); lane 8= Rat CA4 (tolerized with human hepatocytes but not inoculated with HBV); lane 9= Rat CA5 (tolerized with human hepatocytes, not injected with human hepatocytes, inoculated with HBV); lane 10=

Rat CA6 (tolerized with human hepatocytes, not injected with human hepatocytes, inoculated with HBV); where lane 1= 1,000 bp ladder.

FIGURE 13A-C. Photomicrographs of hematoxylin-eosin stained liver sections, at low (20X) magnification, of liver sections from a rat tolerized, transplanted with human hepatocytes, and inoculated with HBV, (13A) 1 week, (13B) 6 weeks, or (13C) 14 weeks post-inoculation.

FIGURE 14A-C. Photomicrographs of hematoxylin-eosin stained liver sections, at high (40X) magnification, of liver sections from a rat tolerized, transplanted with human hepatocytes, and inoculated with HBV, (13A) 1 week, (13B) 6 weeks, or (13C) 14 weeks post-inoculation.

FIGURE 15. Mixed lymphocyte assays. Splenic lymphocytes obtained two weeks after birth were used as responder cells. Irradiated primary human hepatocytes served as stimulator cells. Lane 1, no stimulator cells were mixed with spleen cells from rats injected intrafetally with only saline (without human hepatocytes); lane 2, irradiated human hepatocytes (stimulator cells), alone; lane 3, irradiated human hepatocytes mixed with spleen cells from rats intrafetally injected with only saline; lane 4, irradiated human hepatocytes mixed with spleen cells from rats intrafetally injected with human hepatocytes and subsequently transplanted with human hepatocytes; lane 5, human hepatocytes mixed with spleen cells from rats intrafetally injected with human hepatocytes cells, but without subsequent transplantation with human hepatocytes. All assays were performed in triplicate. The results expressed as means  $\pm$  S.D. in units of cpm/ $10^6$  cells.

FIGURE 16A-F. Immunohistochemical staining of cells for human albumin, left panels, and Hepatitis B Surface Antigen (HBsAg), right panels, of livers 15 weeks after inoculation with purified HBV. Panels A and B, a representative liver section from rats injected intrafetally with primary human hepatocytes, transplanted with primary human hepatocytes shortly after birth, and inoculated with purified HBV 1 week after birth. Panels C and D, rats injected intrafetally with human hepatocytes, transplanted with human hepatocytes, but not inoculated with HBV. Panels E and F, rats that received intrafetal tolerization, no human hepatocyte transplantation, but were inoculated with HBV, under identical conditions. The bars



represent 10  $\mu$ .

FIGURE 17A-D. *In situ* hybridization with a [ $^{32}$ P]-HBV probe. *In situ* hybridization of rat liver was performed as described in section 9.1, Materials and Methods. Panel A, tolerization followed by human hepatocyte transplantation, but no  
5 HBV inoculation. Panel B, tolerization, and no transplantation, but with HBV inoculation. Panel C, tolerization, transplantation, and HBV inoculation. Panel D, tolerization without transplantation or HBV exposure. Magnification 40X:

FIGURE 18A-B. Panel A. Polymerase chain reaction for detection of serum HBV DNA. Lane 1, DNA molecular weight markers; lane 2, serum from  
10 normal rat; lane 3, positive control DNA from HepG2 2.2.15. Lanes 4-6 and 7-9, serum from two representative tolerized rats transplanted with human hepatocytes 1, 5, and 15 weeks after HBV exposure. Lanes 10 and 11, tolerized rats with human hepatocytes alone (no HBV) at 15 weeks. Lanes 12-14 and 15-17, tolerized rats without human hepatocytes, but with HBV exposure at 1, 5, and 15 weeks after HBV  
15 inoculation. Panel B. DNA dot blots of serum DNA. Two normal untreated rats, rows A and B, column 1. Two tolerized rats with human hepatocytes and exposed to HBV, rows A and B, columns 2, 3, and 4 representing post-inoculation weeks 1, 5 and 15, respectively. Tolerized rats transplanted with human hepatocytes, but with no  
20 HBV exposure, rows C and D, column 1 at 15 weeks. Tolerized rats without human hepatocytes, but exposed to HBV, rows C and D, columns 2, 3 and 4 representing post-inoculation weeks 1, 5 and 15 weeks, respectively. Row E, standard HBV DNA in serial dilutions of  $10^6$ ,  $10^5$ ,  $10^4$  and  $10^3$  copies in columns 1, 2, 3, and 4 respectively.

FIGURE 19A-B. PCR detection of cccHBV DNA as evidence of replication. Panel A: Liver. Lane 1, DNA molecular weight markers; lane 2, control  
25 untreated rat; lane 3, DNA from HepG2 2.215 cells, positive control. Lanes 4 and 5: DNA from livers of two tolerized rats with human hepatocytes at 15 weeks after inoculation with HBV. Lane 6, DNA from liver of a tolerized rat with human hepatocytes alone, with no HBV exposure at 15 weeks. Lanes 7 and 8, tolerized rats with no transplantation, but with HBV inoculation, at 15 weeks after inoculation.  
30 Panel B: Serum. Lane 1, DNA molecular weight markers; lane 2, control untreated rat; lane 3, DNA from HepG2 2.215 cells, positive control; lanes 4, DNA from a tolerized

rat with human hepatocytes at 15 weeks after inoculation with HBV; lane 5, DNA from tolerized rat with human hepatocytes alone, with no HBV exposure, 15 weeks post-inoculation; lane 6 tolerized rats, with no transplantation, but with HBV inoculation, at 15 weeks after inoculation.

5                   FIGURE 20. Time course of serum HBsAg. Tolerized rats transplanted with human hepatocytes inoculated with HBV, solid circles; tolerized rats that did not receive human hepatocyte transplantation, but were inoculated with HBV, open squares; and tolerized rats transplanted, but did not receive HBV, crosses.

## 5. DETAILED DESCRIPTION OF THE INVENTION

10                   The present invention relates to tolerized non-human animals having chimeric livers comprising human hepatocytes, methods for preparing such animals, and the use of such animals either as model systems for assaying toxicology or studying human liver disease or as sources of human hepatocytes for re-introduction into a human host. For purposes of clarity, the description of the invention is  
15                   presented as the following subsections:

- i) producing animals having chimeric livers;
- ii) toxicology model systems;
- iii) model systems for liver diseases; and
- iv) chimeric animals as a source of hepatocytes for liver reconstitution.

20                   The subject animals of the invention are referred to herein alternatively as "non-human animals having chimeric livers" or simply "chimeric animals". Both these terms are defined as tolerized non-human animals having livers which comprise human hepatocytes. In addition to the human hepatocytes, the livers of the chimeric animals may also include hepatocytes and non-hepatocyte elements (*e.g.*, biliary and  
25                   vascular endothelial cells, Kupffer cells, etc.) endogenous to the animal itself. Human cell types other than hepatocytes may also be present. Preferably, the percentage of human hepatocytes (relative to the total number of hepatocytes present) is at least 10 percent, more preferably at least 20 percent, or at least 50 percent, or at least 80 percent.

In particular, chimeric animals are created by introducing human hepatocytes (and possibly additional cell types) into an animal rendered immunologically tolerant to the introduced human cells. As such, the animals may be referred to as being "hosts" to the human cells, where a human being that is a source of such cells may be referred to as a "donor". The term "tolerant", as used herein, does not refer to a state of general immunosuppression (as might be achieved, for example, by treatment with cyclosporine, or as may exist in an animal with a generalized B cell and/or T cell deficiency) but rather indicates a state of antigen-induced non-responsiveness of lymphocytes achieved by clonal deletion, cell-mediated suppression, or anergy (see, for example, Davies, 1997, *"Introductory Immunobiology"*, Chapman & Hall, London, p. 366) directed specifically toward the introduced human cells.

#### 5.1. PRODUCING ANIMALS HAVING CHIMERIC LIVERS

The present invention provides for a method of preparing a non-human animal having a liver comprising human hepatocytes, comprising (i) inducing tolerance in a host animal, where the animal is preferably a fetus or a neonate; and (ii) introducing human hepatocytes into the tolerized animal, preferably postnatally and preferably by intra-splenic injection. In specific embodiments, the host animal is subjected to a selection pressure which favors survival and/or proliferation of human, rather than host animal, hepatocytes. A detailed non-limiting description of these features of the invention is set forth in the following subsections.

##### 5.1.1. HOST ANIMALS

Non-human animals which may serve as hosts according to the invention are preferably mammals, and include, but are not limited to, mice, hamsters, rats, rabbits, dogs, goats, sheep, pigs, cattle, etc.. In particular non-limiting embodiments of the invention, the host animal is a transgenic animal carrying, as a transgene, a gene which, when expressed in hepatocytes, is directly or indirectly (i.e. via a metabolite) toxic to those cells. Examples of such genes are the urokinase gene which is directly toxic (Sandgren et al., 1991, *Cell* 66:245), and the *Herpes simplex*

virus ("HSV") thymidine kinase gene ("HSV-TK"); which converts the drug gancyclovir into a toxic form and is therefore indirectly toxic (Smythe et al., 1995, Ann. Surg. 222:78-86). Preferably, the gene is operably linked to a promoter which is selectively active in hepatocytes, such as the albumin promoter, the PEPCK promoter, and the hepatitis B surface antigen promoter. To avoid destroying the animal's liver prior to colonization with human hepatocytes, it is desirable to utilize a promoter that is not particularly active pre-natally. Otherwise, such transgenic animals may die *in utero*. Other promoters inducible by agents that could be locally administered into the liver may also be suitable, such as the metallothionein promoter (which is inducible by heavy metal ions; Palmiter et al., 1982, Cell 29:701). Such genes are not specifically toxic to human hepatocytes, although there may be some "bystander effect" whereby a limited number of the human hepatocytes are killed.

In one specific, non-limiting embodiment of the invention, transgenic mice carrying an albumin promoter/urokinase transgene may be used as hosts. Urokinase is a plasminogen activator that is useful clinically in dissolving blood clots. When introduced into hepatocytes by an adenoviral vector, it was shown to be toxic to those cells (Lieber et al., 1995, Proc. Natl. Acad. Sci. U.S.A. 92:6210-6214). In addition, Sandgren et al. prepared a transgenic mouse containing the mouse urokinase gene driven by a mouse albumin enhancer/promoter (Sandgren et al., 1991, Cell 66:245-256). Because albumin is not produced by the fetal liver (Krumlauf et al., 1985, Cold Spring Harbor Symp. Quant. Biol. 50:371-378), animals survived *in utero* because urokinase was not produced. However, after birth, with activation of the albumin promoter, the liver was destroyed due to the presence of urokinase. To produce such a transgenic mouse for use as a host, heterozygote transgenic mice, B6SJL background, may be obtained from Jackson Laboratories, Stock No. 002214, which contain the mouse urokinase gene driven by a 3.5 kb mouse albumin promoter sequence with a human growth hormone poly A addition site. Pregnant mice from heterozygotic matings may be used to generate homozygous offspring. The number of copies of the urokinase transgene present in each animal at birth may be determined from DNA extracts of tail snips, where the DNA may be digested with Kpn I, which cuts once within the urokinase gene, and Southern blotting using a detectably labeled

probe specific for the urokinase gene, such as 5'- TGTGCTTATG TAGCCATCCA GCGAGTCCCC-3' (SEQ ID NO: 1). Because somatic mutations leading to inactivation of the urokinase gene may occur, it may be desirable to use breeding pairs of male and female mice successfully rescued into adulthood by introduction of human hepatocytes to generate litters of homozygous offspring. Further, in previous studies on mice carrying a urokinase transgene, inactivating mutations in the urokinase gene were found to result in proliferation of those cells with that somatic mutation while the homozygous cells failed to grow. The proliferating cells, as expected, had higher ploidy than those less actively proliferating (Sandgren et al., 1991, *Cell* 66:245-256). Thus, the copy number of human DNA, if measured during proliferation of human hepatocytes may be biased, and not reflect the number of cells due to polyploidy. For this reason, the number of human cells may be better estimated by measuring markers specific for human hepatocytes, such as, but not limited to, the human albumin gene or its protein product.

In another specific non-limiting embodiment of the invention, transgenic mice carrying an albumin promoter/HSV-thymidine kinase gene may be used as hosts. Thymidine kinase of HSV differs from mammalian thymidine kinases in its ability to phosphorylate the drug gancyclovir (Fyfe et al., 1978, *J. Biol. Chem.* 253:8721-8727). In so doing, it converts the non-toxic agent into a toxic form (De Clerq, 1984, *Biochem. Biopharmacol.* 33:2159-2169). In a specific non-limiting embodiment, the HSV-TK gene (as present in plasmid pLTR-DTK, as developed by D. Klatzmann, Université Pierre et Marie Curie, Paris, France) may be linked to an albumin promoter prepared by excising a 3.2 kb fragment of the mouse albumin promoter (for example from *palb<sub>9,12</sub>*LDLR, James Wilson, University of Pennsylvania, Philadelphia, PA) using Bgl II and Sal I restriction enzymes (Wilson et al., 1992, *J. Biol. Chem.* 267:963-967); and placing the promoter fragment in a polylinker site immediately upstream of the HSV-TK gene. Using this plasmid, founder outbred CD1 mice may be prepared and mated to normal CD1 mice to generate heterozygotes, detected by DNA analysis of tail snips using an HSV-TK specific detectably labeled probe. A breeding pair of heterozygotes may then be used to produce mice homozygous for the albumin promoter/HSV-TK transgene. It should be noted that the

natural HSV-TK gene contains elements that activate the gene in the testes, which may result in sterile animals that cannot be used as breeders. Accordingly, a version of the gene which lacks these elements is preferred, such as the gene contained in plasmid pLTR- $\Delta$ TK (all such variant genes, as well as the wild-type, are considered HSV-TK genes). Breeding of transgenic mice with this specific construct confirmed the success of the deletion (Salomon et al., 1995, Mol. Cell. Biol. 15:5322-5328). Further, a gancyclovir dose-related (Culver et al., 1992, Science 256:1550-1552) bystander effect of the HSV-TK gene product has been observed whereby nearby cells lacking the transgene are destroyed (Kolberg, 1994, J. NIH Res. 6:62-64). Accordingly, it may be desirable to evaluate different doses of gancyclovir and identify the minimum dose required to produce maximal human hepatocyte proliferation.

#### 5.1.2. TOLERIZATION

Non-human animals which are to be used as hosts for human hepatocytes may be rendered tolerant to those hepatocytes by administration of the relevant antigen(s), preferably in the context of human cells or a lysate prepared from human cells, more preferably using human cells from the same individual who is to serve as the hepatocyte donor. Tolerizing antigen(s) may be administered as whole cells, a cell extract or one or more purified component thereof. The source of tolerizing antigen(s) may be hepatocytes, but may alternatively be cells of another type, or a mixture of different types of cells. For example, cells prepared from a specimen of human liver tissue may be used as a source of tolerizing antigen(s); such cells may include not only hepatocytes but also fibroblasts, cells of the biliary system, vascular endothelial cells, Kupffer cells, etc.. As another example, human splenocytes or lysates thereof may be used to induce tolerance. Cells to be used in tolerization are preferably cleared of undesirable constituents. For example, if the animal is eventually to be used as a model system for a disease where an immune response to an infectious agent is desirably left intact, the animal should not be tolerized against the infectious agent. Alternatively, if the animal is to be used as a host to support the proliferation of human hepatocytes to be used to reconstitute the liver of a person having liver damage

caused by an infectious agent, it is desirable not to tolerize the host animal toward the infectious agent or to introduce the infectious agent into the host animal at any time. The cells or lysate are introduced in a physiologically compatible solution; herein, volumes administered refer to cells or lysate comprised in such a solution.

5                   While the host animal may potentially be of any age when tolerized, tolerization is likely to become more difficult as age of the animal increases. Preferably, the animal is still an infant when tolerized; more preferably, the animal is a neonate, or tolerized *in utero*. If the intended host animal is a rat, the preferable upper age limit for tolerization is 18 days post-conception (*in utero*), and the more preferable  
10                   age for tolerization is 17 days post-conception (*in utero*), or within 24 hours after birth. If the intended host animal is a mouse, the preferable upper age limit for tolerization is 18 days post-conception (*in utero*), and the more preferable age for tolerization is 17 days post-conception (*in utero*), or within 24 hours after birth. If the intended host animal is a pig, the preferable upper age limit for tolerization is 90 days  
15                   post-conception, and the more preferable age for tolerization is 80 days post-conception, when the animal is still *in utero*, or within 24 hours after birth.

                  Tolerization may be accomplished by any route, including but not limited to intravenous, intraperitoneal, subcutaneous, and intrathymic routes. Preferred methods of tolerization include inoculation of human cells into the thymus  
20                   or intraperitoneally.

                  As a specific, non-limiting example, where the intended host animal is a rat, tolerance may be induced by inoculating lysate prepared from  $1 \times 10^4$  -  $1 \times 10^6$  and preferably  $10^5$  human hepatocytes into the peritoneum of a 15-18 day old, and preferably a 17 day old, rat fetus *in utero* under transillumination. The lysate may be  
25                   prepared by sonicating a suspension of the appropriate number of human hepatocytes. The same numbers of whole cells may also be inoculated into the peritoneum during the aforesaid time periods. If the intended host animal is a mouse, the number of human hepatocytes represented in the lysate may be  $1 \times 10^3$  -  $1 \times 10^5$  and preferably  $10^4$  and intraperitoneal inoculation may be performed between days 15 and 18 post  
30                   conception. If the intended host animal is a pig, the number of human hepatocytes represented in the lysate may be between about  $10^5$  and  $10^6$  or the same number of

whole cells and intraperitoneal inoculation may be performed at between about 75 and 90 days post-conception. Alternatively, intraperitoneal inoculation can be performed while the animals are neonates.

As a second non-limiting example, tolerance may be induced by intrathymic injection according to a method as described in Fabrega et al., 1995, Transplantation 59:1362-1364. Either whole cells or a cell lysate may be administered. In particular, where the intended host animal is a rat, about  $1 \times 10^2$  -  $1 \times 10^5$  human hepatocytes (or a lysate thereof) in between about 1 and 10 microliters, preferably about 5 microliters, may be injected into the thymus of a newborn (neonatal) rat, preferably within 1-2 hours of birth. Where the intended host animal is a mouse, about  $1 \times 10^2$  -  $1 \times 10^4$  and preferably 100 human hepatocytes (or a lysate thereof) in between about 1 and 10 microliters and preferably about 5 microliters may be injected into the thymus of a mouse that is up to 3 months old and preferably a neonate, e.g. within 1-2 hours or within 24 hours of birth. Where the intended host animal is a pig, about  $10^5$  -  $10^6$  human hepatocytes (or a lysate thereof) in between about 50 and 200 microliters may be injected into the thymus of an infant pig that is preferably up to one week old. As a specific example, a neonatal mouse may be anesthetized by chilling on ice, the thoracic area may be cleaned with alcohol and betadine swipes, the thymus may be visualized through the translucent skin of the newborn, and a 1-2 mm incision may be made with ophthalmic scissors to expose the thymus. The human cells or human cell lysate may then be slowly injected into the thymus, and then the incision may be closed with a sterile nylon suture. The incision area may then be recleaned and the mouse placed on a warming pad and returned to its mother as soon as possible.

The success of tolerization may be assessed by proceeding to introduce human hepatocytes into the animal, and determine whether or not they survive long-term (for example, by monitoring the production of human serum albumin; see *infra*). Alternatively, the ability of lymphocytes from the animal to react with donor human hepatocytes may be evaluated using standard immunologic techniques, such as methods that determine T cell proliferation in response to donor hepatocytes, the induction of a cytotoxic T cell response, or mixed lymphocyte reaction.



### 5.1.3. INTRODUCTION OF HUMAN LIVER CELLS

Human liver cells may then be introduced into host animals rendered tolerant as set forth in the preceding section. The hepatocytes may preferably be introduced via intrasplenic injection, although other routes may also be used, such as  
5 direct injection into the liver parenchyma, under the liver capsule, or via the portal vein.

As a specific non-limiting example, where the intended host animal is a rat tolerized as set forth above, between about  $10^6$  -  $5 \times 10^7$  human hepatocytes, preferably about  $2 \times 10^6$  hepatocytes, may be introduced into a tolerized rat within  
10 about 24 hours after birth by anesthetizing the animal, making a 3-4 mm incision in the left paracostal area to visualize the spleen (Marucci et al., 1997, Hepatol. 26:1195-1202), and injecting the donor cells in a volume of approximately about 50-300 microliters, and preferably about 200 microliters, of sterile medium. Where the intended host animal is a tolerized mouse, the number of human hepatocytes  
15 introduced by an analogous procedure may be between about  $5 \times 10^3$  and  $5 \times 10^6$ , preferably about  $10^5$  in a volume of about 25-200 microliters, and preferably about 100 microliters, of sterile medium, and the human hepatocytes are administered between about one day and two months, preferably 3-4 days, after tolerization. Where the intended host animal is a tolerized pig, the number of human hepatocytes may be  
20 between about  $10^8$  -  $10^{10}$ , preferably about  $10^9$ , in a volume of about 10-20 milliliters of sterile medium and the human hepatocytes are administered about one and seven days after birth or about 35 days after tolerization.

Human hepatocytes may be obtained from a commercial source, for example, Clonetics Corporation, 8830 Biggs Ford Road, Walkersville, MD 21793,  
25 which sells normal human hepatocytes as catalog number CC-2591.

Alternatively, human hepatocytes may be prepared from a donor as follows. The source of cells may be from a liver biopsy taken percutaneously or via abdominal surgery, or from liver tissue obtained postmortem. The source of cells should be maintained in a manner which protects cell viability. In one specific non-  
30 limiting embodiment, human hepatocytes may be prepared using the technique described in Guguen-Guillouzo et al., 1982, "High yield preparation of isolated human

adult hepatocytes by enzymatic perfusion of the liver", Cell Biol. Int. Rep. 6:625-628. Briefly, the method of Guguen-Guillouzo et al. involves (i) isolating a liver or a portion thereof from which hepatocytes are to be harvested; (ii) introducing a cannula into the portal vein or a portal branch; (iii) perfusing the liver tissue, via the canula, with a calcium-free buffer followed by an enzymatic solution containing 0.025% collagenase (*e.g.*, Type 4, from Sigma Chemical Company) in 0.075% calcium chloride solution in HEPES buffer at a flow rate of between 30 and 70 milliliters per minute at 37°C; then (iv) mincing the perfused liver tissue into small (*e.g.* about 1 cubic millimeter) pieces; (v) continuing the enzymatic digestion in the same buffer as used in step (iii) for about 10-20 minutes with gentle stirring at 37°C to produce a cell suspension; and (iv) collecting the released hepatocytes by filtering the cell suspension produced in step (v) through a 60-80 micrometer nylon mesh. The collected hepatocytes may then be washed three times in cold HEPES buffer at pH 7.0 using slow centrifugation (*e.g.*, 50 x g for five minutes) to remove collagenase and cell debris. Non-parenchymal cells may be removed by metrizamide gradient centrifugation. If the amount of liver tissue is too small to perform the above perfusion procedure, for example, less than 100 g of tissue, then the tissue may be minced and digested with collagenase solution with gentle stirring and processed according to steps (iv) and (v) of this paragraph.

It may be desirable to separate human hepatocytes prepared as set forth above into a subset for introduction into animals and another subset which is undesirable to propagate. For example, if a human subject is to serve as a donor for hepatocytes which are to be propagated in a chimeric animal according to the invention and then reintroduced into the subject, *e.g.*, to reconstitute a liver damaged by infectious disease or malignancy, it would be desirable not to propagate hepatocytes which are infected or which have undergone malignant transformation. In such a situation, it would be desirable to eliminate infected or malignant hepatocytes from the population of hepatocytes which is to be introduced into the host animal. Elimination of unwanted cells can be performed by standard cell sorting techniques, for example fluorescence activated cell sorting using an antibody specific for the infectious agent or for malignant transformation. Alternatively, undesirable cells may

be eliminated or attenuated by treatment with antiviral or antimicrobial compounds, radiation, antibody-ligated toxins, culture techniques, etc..

#### 5.1.4. FAVORING PROLIFERATION OF HUMAN HEPATOCYTES

In particular non-limiting embodiments of the invention, selection  
5 pressure may be used to favor the proliferation of human hepatocytes. Such selection pressure is defined herein as including any condition, preexisting in the host animal at the time of introduction of donor cells or imposed thereafter, which results in a greater likelihood that human hepatocytes, rather than host hepatocytes, will proliferate.

For example, the selection pressure may result from the presence of a  
10 transgene that decreases the viability of host hepatocytes, either intrinsically (directly) or by administration of an activating agent (indirectly). Alternatively, human donor hepatocytes can be transfected with a protective gene that will enable those cells to survive subsequent exposure to a hepatotoxin. In one specific non-limiting example, the transgene may be the albumin promoter/urokinase construct, whereby as the host  
15 animal matures and the albumin promoter becomes active, host hepatocytes may be eliminated by the toxic effects of urokinase. In such cases, the selection pressure is maturation of the animal with consequent transgene activation. In a second specific non-limiting example, the transgene may be the albumin promoter/HSV-TK construct, whereby when gancyclovir is administered to the host animal (*e.g.*, as an  
20 intraperitoneal injection of 250 mg/kg gancyclovir in sterile PBS), hepatocytes of the transgenic host may be selectively killed. In such embodiments, the death of host hepatocytes would be expected to favor compensatory proliferation of human hepatocytes. This can occur because of the known property of parenchymal liver cells to proliferate during conditions that stimulate regeneration.

25 It may be preferable to effect stepwise attenuation of host hepatocytes rather than eliminate a majority in a short period of time, as the sudden loss of liver function could result in death of the animal and/or conditions that would disfavor the establishment of a human hepatocyte population in the host liver. For example, administration of several doses of gancyclovir to a host animal transgenic for the  
30 albumin promoter/HSV-TK construct, beginning before and continuing after

introduction of donor cells, may result in a gradual elimination of host cells, thereby permitting human hepatocytes to establish a "foothold" before the majority of host hepatocyte function is eliminated.

In another non-limiting embodiment, donor hepatocytes can be  
5 transfected with a protective gene. For example, a gene encoding an antisense RNA or ribozyme against the cytochromes 2E1, 1A2, and/or 3A4 (CYP2E1, CYP1A2, CYP3A4, respectively), would prevent activation of the drug acetaminophen. Metabolites of that agent within liver cells results in hepatocyte death. Thus, donor cells containing the transgene would have a survival advantage relative to host cells if  
10 massive doses of acetaminophen were administered after cell transplantation. A similar strategy would be to transfect a mutant RNA polymerase II that is resistant to the effects of the hepatotoxin phalloidin. Administration of phalloidin to hosts bearing transfected human hepatocytes producing the mutant polymerase would be protected and have a selective advantage over host cells.

#### 15 5.1.5. CONFIRMING THE PRESENCE OF HUMAN HEPATOCYTES

The presence of human hepatocytes in a host may be evaluated by assaying for specific human markers. The presence of such markers in a blood sample or a liver biopsy collected from the animal (*e.g.*, percutaneously) may be evaluated without affecting the viability of the animal. Alternatively, the success of  
20 chimerization may be evaluated retrospectively at necropsy.

As a specific example, the presence or absence of immunologically distinct human albumin may be determined in a blood or tissue sample by Western blot analysis or immunohistochemistry using antibody specific for human, but not host, albumin (see, for example, Wu et al., 1991, J. Biol. Chem. 266:14338-14342;  
25 Osborn and Weber, 1982, Meth. Cell Biol. 24:97-132). An example of a publicly available antibody specific for human albumin is Sigma #A6684 monoclonal anti-human albumin HSA II.

## 5.2. TOXICOLOGY MODEL SYSTEMS

In particular non-limiting embodiments of the invention, a chimeric animal prepared as set forth above may be used as a model system for human hepatocyte function in a toxicology study to determine the toxic effect(s) of a test agent on (i) the human hepatocytes present in the animal and/or (ii) the host animal itself. The chimeric animals of the invention provide the opportunity to recapitulate, in a model system, metabolism of the test agent by human hepatocytes, which may result in one or more secondary compounds that may not be produced when the test agent is exposed to non-human hepatocytes.

Because a test agent may have different effects on host hepatocytes and human hepatocytes, it is desirable to determine the relative proportion of human and host hepatocytes in each test animal, for example by quantitation of the amounts of human and non-human albumin in a serum sample. The ability of this measurement to accurately reflect liver cell populations may be established by correlating serum levels with hepatocyte populations as evaluated by immunohistochemistry in liver tissue samples obtained by biopsy or at necropsy. Once the relative proportions of hepatocyte populations for each animal are determined, experimental results relating to the effect of test agent may be compared with the effect of test agent on a control non-chimeric animal which represents a population of 100 percent host hepatocytes. Preferably, the host hepatocytes are less sensitive to test agent than human hepatocytes.

Accordingly, chimeric animals of the invention may be used to evaluate the toxic effect(s) of a test agent on the viability (survival, function) of human hepatocytes in the animal and/or the animal as a whole by subjecting at least one and preferably a plurality of chimeric animals and non-chimeric animals of the same species (as controls) to incremental doses of test agent. At one or a series of time point(s), the animal(s) may be evaluated by standard laboratory tests to determine whether toxic effects have occurred. Such an evaluation may include an assessment of bodily functions, as reflected by weight and/or activity and analysis of blood and/or urine, for example for test agent or its metabolites, markers of liver function and/or hepatocyte viability, kidney function, immune function, etc.. As discussed above, such

information is considered in view of the percentage of human hepatocytes in each test animal's liver and the relative effects of test agent on human versus host hepatocytes. Further, the percentage of human hepatocytes may change during the course of an experiment, for example, if the test agent is selectively toxic to human hepatocytes so that compensatory proliferation of host hepatocytes occurs. Accordingly, it is desirable to perform measurements of relative quantities of one or more marker specific for human hepatocytes at each time point; for example, the relative amounts of human and host albumin in serum may be measured by Western blot. At one or more time point of the study, an animal(s) may be biopsied and analyzed for human versus host albumin gene or gene product, or human-specific Alu repeat sequence, or sacrificed and a complete necropsy analysis be performed, including immunohistochemical evaluation of hepatocyte populations in the liver.

### 5.3. MODEL SYSTEMS FOR LIVER DISEASES

In another non-limiting embodiment of the invention, an animal having a chimeric liver may be used as a model system for human liver disease. Such chimeric animals may be used to create models of liver disease resulting from exposure to a toxin, infectious disease or malignancy. The model systems of the invention may be used to gain a better understanding of these diseases and also to identify agents which may prevent, retard or reverse the disease processes.

Where the chimeric animal is to be used as a model for liver disease caused by a toxin, animals prepared as set forth above may be allowed to mature to a point where the size of the human hepatocyte population is substantial (e.g. has approached a maximum), and then be exposed to a toxic agent. The amount of toxic agent required to produce results most closely mimicking the corresponding human condition may be determined by using a number of chimeric animals exposed to incremental doses of toxic agent. Examples of toxic agents include but are not limited to alcohol, acetaminophen, phenytoin, methyldopa, isoniazid, carbon tetrachloride, yellow phosphorous, and phalloidin.

In embodiments where a chimeric animal is to be used as a model for malignant liver disease, the malignancy may be produced by exposure to a

transforming agent or by the introduction of malignant cells. The transforming agent or malignant cells may be introduced with the initial colonizing introduction of human hepatocytes or, preferably, after the human hepatocytes have begun to proliferate in the host animal. In the case of a transforming agent, it may be preferable to administer the agent at a time when human hepatocytes are actively proliferating. Examples of transforming agents include aflatoxin, dimethylnitrosamine, and a choline-deficient diet containing 0.05-0.1% w/w DL-ethionine (Farber and Sarma, 1987, in *Concepts and Theories in Carcinogenesis*, Maskens et al., eds, Elsevier, Amsterdam, pp. 185-220). Such transforming agents may be administered either systemically to the animal or locally into the liver itself. Malignant cells may preferably be inoculated directly into the liver.

Where the chimeric animal is to be used as a model for infectious liver disease, the infectious agent, or an appropriate portion thereof (e.g. a nucleic acid fragment) may be introduced with the initial introduction of hepatocytes or after the human hepatocytes have begun to proliferate. The infectious agent may be administered as a free entity or incorporated into a human cell such as a human liver cell. Examples of infectious diseases suitable for modeling include but are not limited to hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatitis E, malaria, Epstein Barr infection, cytomegalovirus infection, and Yellow Fever. For such models, it may be advantageous that the host animal has an immune system that is intact (but for the induced tolerance to the host cells), in that the animal's immune response to the infectious agent and/or infected human hepatocytes may produce a more accurate model of human liver diseases in which the immune system plays a pathogenic role. As such, it may be desirable to ensure that the cells/cell lysate used for tolerization not include infectious agent or related antigens. A working example in which the invention is used to produce a hepatitis B virus model system is set forth below.

Further, where the infectious agent is a virus, the present invention provides for chimeric animals comprising human hepatocytes that contain a nucleic acid of the virus, such as the entire viral genome or a portion thereof, or a nucleic acid encoded by the viral genome or a portion thereof.

### 5.3.1. HCV MODEL PRODUCED BY INFECTIOUS SERUM

In a particular non-limiting embodiment, the invention provides for a chimeric animal model for hepatitis C virus infection. Preferably, the chimeric animal is a mouse transgenic for a gene whose product is selectively toxic to hepatocytes, such as the albumin promoter/urokinase gene or the albumin promoter/HSV-TK gene. Hepatitis C infection of human hepatocytes in such mice may be produced either (i) concurrently with or preferably (ii) after the colonizing introduction of human hepatocytes and after the effects of the toxic transgene have attenuated or eliminated host hepatocytes. Preferably, the chimeric animal has, prior to infection, a liver which comprises substantially (at least about 20 percent, preferably at least 50 percent, more preferably at least 80 percent) human hepatocytes.

The source of infectious agent may be serum from one or more human subject infected with HCV but not demonstrably infected with one or more other agents that infect hepatocytes. Serum samples of genotype 1a may be assayed for viral load by branched DNA (bDNA) assay (Chiron, San Francisco, CA). Sera from non-infected subjects and individuals with non-viral hepatitis may be used to pseudo-infect control chimeric animals. Using standard biohazard precautions, serum containing HCV RNA in infectious human serum, at a titer ranging between about  $10^3$  -  $10^7$  particles per milliliter may be injected intravenously into a chimeric transgenic mouse about 2-4 months and preferably about 6 weeks after colonization with human hepatocytes. Preferably, increasing amounts of HCV RNA in infectious human serum, with the viral titer previously determined (e.g., by National Genetics Institute, Los Angeles, CA ) may be injected into a panel of such chimeric transgenic mice. The site of injection may be the tail vein, and the volume of serum injected may be 0.1 -0.5 ml. The serum may preferably be filter sterilized prior to administration.

Serum may be collected from the chimeric mouse (mice) and tested to establish baseline and post-infection levels of liver function markers such as AST (aspartate amino transferase), ALT (alanine aminotransferase) and alkaline phosphatase. For example, baseline and weekly post-infection levels of AST, ALT and alkaline phosphatase in mouse sera may be determined spectrophotometrically using kits from Sigma Chemical Co., St. Louis, MO, where appropriate standards are



used to generate reference curves. Blood samples may be obtained from the animals retroorbitally using standard techniques.

The mouse (mice) may be tested for seroconversion against HCV by testing for circulating antibody (*e.g.*, anti-C100-3 antibody), for example using the  
5 ELISA kit available from Ortho Diagnostics (catalog number 930740: Ortho HCV ver. 3.1 ELISA TEST SYSTEM; Ortho Diagnostics, Raritan, NJ). Tests for seroconversion may be performed, for example, at weekly intervals for the first month after infection and then monthly.

Viral load may be determined (*e.g.*, weekly) by assay of dilutions of  
10 serum for positive strand HCV RNA using thermostable rTth RT-PCR performed under stringent conditions (at 70° C) to eliminate false priming of the incorrect strand. Branched DNA analysis may also be used, but it is not as sensitive. For positive strand RNA analysis, the cDNA reverse primer may be: 5'-TCGCGACCCA ACACTACTC  
3' (SEQ ID NO: 2) and the forward primer may be 5'-GGGGGCGACA  
15 CTCCACCA-3' (SEQ ID NO: 3). PCR amplification in the absence of reverse transcriptase activity may be accomplished by chelating manganese and magnesium ions as described in (Lanford et al., 1995, J. Virol. 69:8079-8083). The amplified product, which spans nucleotides 15-274 of the 5'-NTR of HCV may be quantitated by Southern blotting using a detectably labeled probe against a region internal to the  
20 primers.

Liver tissue obtained by biopsy or from a sacrificed animal may be evaluated for HCV replication and for histopathological changes. Biopsy may be performed by anesthetizing the chimeric mouse with intramuscular injections of  
25 ketamine (40 mg/kg) and xylazine (5 mg/kg), cleaning the abdominal area with alcohol and betadine wipes, making a 1 cm incision in the abdominal wall to expose the liver, and collecting a sliver (approximately 10 mg) of liver tissue. Afterward, 100U of sterile thrombin may be administered locally at the biopsy site followed by application of gel foam to inhibit bleeding, the abdominal wall may be closed with dissolvable sutures, and the skin may be closed with nylon sutures. Viral replication  
30 may be quantitated by measuring the amount of negative strand template HCV RNA in liver RNA (prepared, for example, as set forth in Chomczynski and Sacchi, 1987,

Anal. Biochem. 162:156-159), using rTth RT-PCR (Lanford et al., 1995, J. Virol. 69:8079-8083). To assess liver histology, liver tissue may be fixed and sectioned and stained with hematoxylin-eosin or trichrome to evaluate, respectively, inflammation or fibrosis. A standardized scoring method, such as Knodell scoring (Knodell et al.,  
5 1981, Hepatology 1:531), may be used. The presence or absence of neoplastic lesions may be evaluated.

To determine the optimum conditions for producing an HCV infected chimeric animal, the time course of serum aminotransferases AST and ALT, alkaline phosphatase levels, and viral RNA loads may be plotted as a function of time and the  
10 minimum number of viral equivalents required to sustain an infection determined. Levels of detectable HCV RNA in the serum of an animal may be used as an indicator of the chronicity of infection.

Potential problems associated with the foregoing embodiment are as follows. First, the detection of negative strand HCV template as a measure of HCV  
15 replication may be problematic due to the requirement for amplification techniques and the possibility of inadvertent amplification of positive strand. The method of Lanford et al. (*supra*) using stringent conditions for priming of the RT-PCR and inactivation of the reverse transcriptase by chelation prior to PCR of the cDNA has been shown to reduce false amplification to  $1/10^4$  -  $1/10^5$ . Second, the fact that mouse  
20 hepatitis virus may be found even in "pathogen free" environments makes it desirable to confirm that host animals are free of the virus, for example using a mouse virus screen as available from Microbiological Associates, Inc., Rockville, MD. (Carlson et al., 1989, J. Clin. Invest. 83:1183-1190), where animals testing positive are not used as hosts. Third, infection may be improved by increasing the amount of human serum  
25 used in the inoculum.

The foregoing description may be applied to nontransgenic mice or other animals and may be adapted, by altering volume of inoculum and time between colonization and inoculation proportionately, to larger animal model systems.

### 5.3.2. HCV MODEL PRODUCED BY INFECTIOUS PLASMID

In a related embodiment, infection may be introduced by HCV plasmid (Kolykhalov et al., 1997, Science 277:570-574) complexed to a liver-specific protein carrier, such as AsOR-PL or AsORlysine-VSVG, where AsOR-PL is asialoorosomucoid polylysine and AsORlysine-VSVG is asialoorosomucoid covalently linked to L-lysine methyl ester and a synthetic 25 amino acid peptide of the VSVG protein. The DNA-protein complex may be formed by slowly adding protein conjugate in 25 microliter aliquots to DNA in 0.15M NaCl with continuous vortexing at room temperature. After 30 minutes of incubation at room temperature absorption at 260 nm, 340 nm and 400 nm may be measured to detect complex formation. Complexes may be filter sterilized by passage through a 0.22 micron filter. About 10 micrograms of the DNA/protein complex in 0.5 milliliters sterile saline may then be injected into the tail vein of a mouse.

### 5.3.3. HCV MODEL PRODUCED BY TRANSPLANTING INFECTED HEPATOCYTES

As an alternative to producing HCV infection by inoculation with infected serum, infection may be produced by transplanting HCV infected hepatocytes into a chimeric animal. Although the infected hepatocytes may be introduced during colonization with human cells, it is preferred that they be introduced into chimeric livers having a substantial population of human hepatocytes. Preferably, the chimeric animal is a mouse transgenic for a gene whose product is selectively toxic to hepatocytes, such as the albumin promoter/urokinase gene or the albumin promoter/HSV-TK gene.

Infected human hepatocytes may be obtained as described in Lieber et al., 1996, J. Virol. 70:8782-8791. Using appropriate pathogen - containment procedures, human liver specimens may be obtained from HCV-infected liver transplant recipients. An apical piece of liver covered on three sides by capsule may be perfused with buffer without calcium and then with collagenase in perfusion buffer with calcium. Hepatocytes may then be pelleted by low speed centrifugation. Non-parenchymal cells may be separated from parenchymal hepatocytes by metrizamide gradient centrifugation. The viability of isolated hepatocytes may be evaluated by

trypan blue exclusion. Hepatocytes may be resuspended in Williams medium at about  $10^7$  cells per milliliter.

The infected hepatocytes may then be introduced into the liver of a chimeric animal such as a chimeric transgenic mouse having a liver which comprises substantially (at least about 20 percent, preferably at least 50 percent, more preferably at least 80 percent) human hepatocytes. The infected hepatocytes may be introduced by intrasplenic injection. Where the animal is a mouse, hepatocytes may be achieved by anesthetizing the animal with ketamine (90 mg/kg) /xylazine (10 mg/kg), and then, under aseptic conditions, making a 2-3 millimeter incision in the left paracostal area, exposing the spleen. The spleen may then be exteriorized and infected hepatocytes may be injected slowly into the spleen parenchyma. Gel foam may be used to achieve hemostasis, the spleen may be restored into the body cavity, and the wound may be sutured closed. Monitoring of the resulting infected animals for seroconversion, viral load, serum levels of protein markers of liver function, and histopathology may be performed as described in section 5.3.1.. Further, these methods may be applied to nontransgenic mice and may be adapted for use in larger animals.

#### 5.3.4. USE OF HCV MODELS

Chimeric animal models of HCV infection may be used not only to study the biology of HCV, but also to identify agents that may prevent or inhibit HCV infection and/or replication. For example, to determine whether a test agent inhibits infection by HCV, the effect of the agent on preventing infection when administered prior to or contemporaneously with injection of infected serum may be evaluated. Similarly, the effect of a test agent administered during the course of infection may be assessed. Parameters useful in determining the effectiveness of test agent would include whether and when the test animal seroconverts with respect to HCV, the viral load, the ability of serum from the animal to infect other animals, blood levels of proteins/enzymes associated with liver function and/or hepatocyte viability, and liver histology.

#### 5.4. CHIMERIC ANIMALS AS A SOURCE OF HEPATOCYTES FOR LIVER RECONSTITUTION

The present invention further provides for the use of chimeric animals as a source of human hepatocytes for liver reconstitution in a second host subject.

- 5 Such reconstitution may be used, for example, to (i) produce a "next generation" chimeric non-human animal; (ii) introduce genetically modified hepatocytes for "gene therapy" of the second host subject; or (iii) replace hepatocytes lost as a result of disease, physical or chemical injury, or malignancy in the second host. Human hepatocytes collected from a chimeric animal are said to be "passed".

- 10 For any of these applications, liver tissue from a chimeric animal may be used to produce a cell suspension and then human hepatocytes may be separated from non-human hepatocytes and other cells. The liver tissue may be processed as set forth above to produce a suspension of hepatocytes. As a non-limiting specific example, where the chimeric animal is a mouse or rat, hepatocytes may be prepared by
- 15 the following method, adapted from Seglen, 1976, "Preparation of rat liver cells", *Methods Cell Biol.* 13:29. Briefly, a chimeric mouse or rat may be anesthetized with ketamine/xylazine, its abdomen may be shaved and decontaminated, the peritoneal cavity may be opened by incision, the inferior vena cava may be cannulated, the portal vein may be divided and the suprahepatic vena cava may be ligated. Then, the liver
- 20 may be perfused *in situ* with calcium free balanced salt solution at 5 ml/min for five minutes at 37°C, followed by perfusion with 0.05% collagenase (*e.g.*, type IV, from Sigma Chemical Co.) in 1% albumin and balanced salt solution for 20 minutes. The liver may then be transferred to a Petri dish, and minced to produce a cell suspension, from which hepatocytes may be collected by passage through a 60-80 micron nylon
- 25 mesh. The collected cells may then be washed three times in RPMI 1640 or Williams E medium with 10% fetal bovine serum, and then centrifuged at 35 x g for five minutes at 4°C. Hepatocytes may be purified through a metrizamide gradient and resuspended in RPMI 1640 or Williams E medium.

- 30 Human hepatocytes may be separated from non-human cells using fluorescence activated cell sorting techniques and an antibody which selectively binds to human hepatocytes, for example but not by way of limitation, an antibody that

specifically binds to a class I major histocompatibility antigen. Suitable antibodies would include but not be limited to anti-human HLA-A,B,C, Pharmingen catalogue # 32294X or #32295X, FITC mouse  $\kappa$ b, PharMingen catalogue #06104D (PharMingen, San Diego, CA) See, for example, the procedure described in Markus et al., 1997, Cell Transplantation 6:455-462.

Human hepatocytes may be passaged through cell transplantation of tolerized host animals, using the techniques set forth above. In this manner, cells obtained from an initial human donor may be utilized in a multitude of chimeric animals and over an extended period of time, potentially reducing the variability that may be encountered in chimeric animals produced using hepatocytes obtained from diverse hosts.

Passaged human hepatocytes may also be used for gene therapy applications. In the broadest sense, such hepatocytes are transplanted into a human host to correct a genetic defect. The passaged hepatocytes need not, but are preferably derived originally from the same individual who is to be the recipient of the transplant. However, according to the invention, hepatocytes from a different individual may alternatively be used.

As a specific, non-limiting example, a patient suffering from intermittent acute porphyria, caused by a genetic defect in the expression of uroporphyrinogen I synthase, may benefit from transplantation of human hepatocytes harvested from a chimeric animal of the invention, where the transplanted cells are genetically normal in their expression of that enzyme. The recipient would be "matched" for transplantation antigens with the original donor, or be treated with immunosuppressive therapy. For such applications, chimeric animals prepared from a wide diversity of individual donors could provide the advantage of constituting a "living library" of differentiated hepatocytes having various transplantation antigen profiles, thereby obviating the need for waiting until liver tissue from a genetically suitable donor becomes available.

Preferably, however, the original donor and eventual recipient of passaged hepatocytes are the same person, thereby eliminating the need for immunosuppression. For gene therapy applications, (i) hepatocytes may be harvested

from the subject, (ii) the desired genetic construct may be introduced into those hepatocytes, (iii) the resulting genetically engineered human hepatocytes may be used to tolerize a host animal to their presence, (iv) construct-carrying hepatocytes may be introduced into the tolerized animal such that its liver is colonized, and then, once  
5 expanded in number, (v) the transgenic hepatocytes may be harvested from the chimeric animal and (vi) reintroduced into the subject. A genetic construct may be introduced into the human hepatocytes by any standard method, including, but not limited to, transfection with naked DNA, microparticles or liposomes, or infection with a viral vector, such as an adenoviral vector, an adeno-associated vector, or a  
10 retroviral vector. Hepatocytes used for colonization may be enriched for cells containing the desired construct, for example, by selection by culture conditions, antibody/FACS methods, etc. which eliminate cells lacking the construct.

Alternatively, the hepatocytes may be used to colonize the liver of a tolerized animal prior to or contemporaneous with the introduction of the desired  
15 transgene via a gene therapy vector. This approach may be more problematic because the host animal could develop an immune response directed toward either the vector or vector-transformed hepatocytes.

In further embodiments, human hepatocytes passaged through a chimeric animal of the invention may be used to reconstitute liver tissue in a subject  
20 as a prelude or an alternative to liver transplant. As a specific non-limiting example, a subject suffering from progressive degeneration of the liver, for example, as a result of alcoholism, may serve as a donor of hepatocytes which are then maintained, through one or several generations, in one or more chimeric animal. As a result of maintenance in such animal(s), the number of hepatocytes is expanded relative to the  
25 number originally harvested from the subject (it may be preferable to use larger animals to produce greater numbers of cells). At some later date, when the subject's liver has deteriorated to a medically hazardous condition, hepatocytes passaged in the chimeric animal(s) may be used to reconstitute the subject's liver function. As a second non-limiting example, passaging hepatocytes may be used not only to expand  
30 the number of hepatocytes but also to selectively remove hepatocytes that are afflicted with infectious or malignant disease. Specifically, a subject may be suffering from

hepatitis, where some but not all of the hepatocytes are infected and infected hepatocytes can be identified by the presence of viral antigens on the cell surface. In such an instance, hepatocytes may be collected from the subject, and non-infected cells may be selected for passaging in one or more chimeric animal, for example by  
5 FACS. Meanwhile, aggressive steps could be taken to eliminate infection in the patient. Afterward, the subjects liver tissue may be reconstituted by hepatocytes passaged in a chimeric animal. An analogous method could be used to selectively passage non-malignant cells from a patient suffering from a primary or secondary (*e.g.* metastatic) liver malignancy. Thus, the chimeric animals of the invention may be  
10 used as a means of purging unwanted hepatocytes from a human subject.

## 6. EXAMPLE: PREPARATION OF RATS HAVING CHIMERIC LIVERS

### 6.1. SURVIVAL OF HUMAN HEPATOCYTES IN RATS TOLERIZED BY INTRAPERITONEAL INJECTION

15 To tolerize hosts for hepatocyte transplantation, human hepatocytes obtained from Clonetics Corp. were suspended at a concentration of  $10^6$  cells/ml saline and sonicated. Laparotomies were performed to expose the gravid uteri of pregnant rats and sterile filtered sonicates equivalent to  $10^4$  hepatocytes in  $10 \mu\text{l}$  were injected into groups of three fetuses each. A control received only the same volume of  
20 normal saline. Within 24 hrs of birth, transplantation of hepatocytes were performed by the method of Marucci et al., 1997, Hepatol. 26: 1195-1202. An incision was made at the left paracostal area, the spleen was visualized and  $2 \times 10^7$  hepatocytes in  $100 \mu\text{l}$  sterile medium were injected slowly into the spleen. To evaluate the status of immune tolerance, two experiments were performed: a) mixed lymphocyte assays and  
25 b) repeat transplantation challenges.

### 6.2. MIXED LYMPHOCYTE ASSAYS

For mixed lymphocyte assays, spleens from intrafetally injected animals were removed six weeks after birth, and spleen cells were isolated as described by Henry and Watson (1980, "Preparation of Spleen Cells" section 2.9, in *Selected Methods in*



*Cellular Immunology*, Mishell and Shiigi, eds., WH Freeman and Co., p.65).

Assays were performed according to the method of Bradley (1980, "Mixed Lymphocyte Responses" section 6.3, in *Selected Methods in Cellular Immunology*, Mishell and Shiigi, eds., WH Freeman and Co., p. 162) in which spleen cells were  
5 used as responder cells and human hepatocytes (identical to those transplanted but treated with 2000R of X-irradiation to prevent proliferation) were used as stimulator cells. Human hepatocytes should not stimulate lymphocyte proliferation in spleen cells from immunotolerant animals, but should in cells from non-tolerant animals.

Spleen cells,  $7 \times 10^5$  cells per assay, from fetuses previously injected  
10 intraperitoneally as described above with hepatocyte lysates alone, lysates followed by hepatocytes after birth, or controls were mixed with  $3 \times 10^5$  X-irradiated simulator hepatocytes. Controls consisted of spleen cells from saline treated fetuses alone, and those same cells plus irradiated spleen cells from non-treated animals. Cells were pulsed with  $1 \mu\text{Ci}$  [ $^3\text{H}$ ]-thymidine (specific activity 5 Ci/mmol) at  $37^\circ\text{C}$  for 72 hours,  
15 and then harvested by TCA precipitation onto Whatman glass fiber filters, washed and scintillation counted. All assays were performed in quadruplicate, and the entire experiment done in duplicate. The results are expressed as means  $\pm$  S.D. in units of cpm/ $10^6$  responder cells in FIGURE 1.

FIGURE 1 shows that spleen cells from animals that did not receive  
20 hepatocyte lysate when they were fetuses incorporated approximately 5,200 cpm/ $10^6$  cells when stimulated with irradiated human hepatocytes. In contrast, stimulation of spleen cells derived from animals that did receive human hepatocyte lysate when they were fetuses was significantly less (less than 1000 cpm/ $10^6$  cells [ $p < .002$ ]) than that observed using cells from animals that did not receive lysate. In fact, cells derived  
25 from animals injected with lysates resulted in stimulation that was no more than background levels of spleen cells alone. Irradiated hepatocytes alone (without spleen responder cells) had no significant radioactive incorporation confirming that the irradiation substantially blocked any contribution to the observed radioactive uptake.

These data indicate that spleen cells removed 6 weeks after birth from  
30 rats previously injected as fetuses with human hepatocyte lysates are not significantly stimulated to proliferate by the presence of human hepatocytes. At this time point,

human albumin is still strongly detectable in serum in the tolerized animals. Together, the data indicate that immune tolerance to human hepatocytes was achieved.

### 6.3. RECHALLENGE WITH ADDITIONAL HEPATOCYTE TRANSPLANTATIONS

5           As further evidence for immune tolerization, groups of rats previously tolerized and transplanted as described above were subjected to a repeat transplantation. If the rats were not rendered immunologically tolerant to human hepatocytes, a second transplantation of human cells would be expected to evoke an anamnestic response and rapid rejection of those cells. To evaluate that possibility,  
10       rats tolerized intrafetally and transplanted with human hepatocytes at birth as described above were given a repeat transplantation of  $2 \times 10^6$  cells 6 weeks after the first. Serum was assayed for human serum albumin using a specific anti-human albumin antibody and analyzed by Western blots as shown in FIGURE 2.

          In FIGURE 2, lane 1 contains 40 ng of human serum albumin standard,  
15       and lane 2 shows that there was human albumin still present in substantial concentration in serum at 6 weeks after an initial cell transplantation. After a second injection of human cells the amount of serum albumin increased 24 hrs later (lane 3), and continued to rise at least 8 days after the repeat dose of cells (lane 4). Animals that had not been tolerized (received only a fetal injection of saline) had no detectable  
20       human serum albumin even after a repeat transplant of human hepatocytes under identical conditions and assayed at the same time point (8 days; lane 5). These data suggest that human hepatocytes did not survive in non-tolerized animals. In contrast, in those animals that were tolerized, human hepatocytes not only survived each of two successive inoculations, but also maintained function as evidenced by serum albumin  
25       production.

### 6.4. SURVIVAL OF HUMAN HEPATOCYTES

Seventeen day old normal Sprague-Dawley rat fetuses were given lysates of  $0.5 \times 10^5$  human hepatocytes by transuterine inoculation into the peritoneum under trans-illumination. Following birth,  $2 \times 10^5$  normal human

hepatocytes were injected into the spleen. This is known to result in near total migration of transplanted hepatocytes to the liver (Attavar, et al., 1997, Hepatol. 26: 1287-1295). At weekly intervals, animals were bled via their tail veins and human serum albumin was detected as a function of time by Western blot analysis using a specific affinity purified rabbit anti-human albumin antibody (Figure 3). At the conclusion of the study, animals were sacrificed and liver slices stained with anti-human albumin antibody and developed with a Texas red secondary antibody. Cells were visualized using a Zeiss confocal scanning microscope, model CLSM4 10 and images were captured as shown in FIGURE 4.

FIGURE 3 shows a representative Western blot of the collected rat sera, where lane 1 contains 10 ng standard human serum albumin, lane 2 contains 10 ng standard rat albumin, lane 3 contains rat serum 1 week after intrasplenic injection into the tolerized rat (1 day after birth), lane 4 contains serum from the same rat as lane 3 but after 2 weeks, lane 5 same as lane 3, but after 3 weeks. Lane 1 shows that the anti-human albumin antibody reacted with standard human serum albumin, but not with rat albumin, (lane 2) (nor mouse albumin, data not shown). Lanes 3, 4, and 5 showed bands corresponding in mobility to albumin detectable at about 10 ng/50  $\mu$ g total serum protein. This level remained stable or increased through at least week 3. Transplantation of human IMR-90 fibroblasts under identical conditions failed to produce any detectable human serum albumin.

FIGURE 4A-D shows a representative immunofluorescence study of a liver section taken from 1 of 4 rats 3 weeks after injection with human hepatocyte lysate *in utero*, followed by intrasplenic injection of human hepatocytes. Immunocytochemistry was performed with primary antibody for human albumin, or rat albumin as a control, and Texas red-coupled secondary antibody. Panel A shows anti-human albumin antibody staining of rat liver without human hepatocyte transplantation. Panel B shows rat livers 3 weeks following injection with human hepatocyte lysate *in utero* and intrasplenic injection of human hepatocytes (1 day after birth) stained with anti-human albumin antibody and Texas Red second antibody. Panel C shows the same section as depicted in B, but without second antibody. Panel D shows a section of control rat liver after only transuterine injection of human

hepatocyte lysate developed with anti-human albumin antibody and Texas Red second antibody. Anti-human albumin staining of liver transplanted with human hepatocytes, but without prior injection with hepatocyte lysate was essentially the same as that shown in Panel A. There was no anti-human albumin staining of normal (non-transplanted rat liver) in Panel A. Cells with fluorescent cytoplasm are seen in Panel B, after both *in utero* lysate injection and human hepatocyte transplantation. This staining was not due to intrinsic fluorescence as there was no signal without second antibody as shown in Panel C. Finally, Panel D shows that the fluorescence could not be due to human albumin from the hepatocyte lysate alone. All 3 other animals injected with human hepatocyte lysate *in utero* and intrasplenic injection of human hepatocytes showed similar results.

FIGURE 5 is a photomicrograph of the same section of rat liver as depicted in FIGURE 4B, 3 weeks after intrasplenic injection of human hepatocytes, here stained with hematoxylin and eosin. The human hepatocytes cannot be distinguished from the rat cells, and there appears to be no inflammation or other evidence of rejection.

FIGURE 6A-D shows the results of an immunofluorescence study performed six weeks after cell transplantation in tolerized animals. Aggregates of cells staining positive for human albumin were present in a tolerized rat that had received a human hepatocyte transplant, panel A. The same section without second antibody, panel B; or no antibody at all failed to produce a fluorescent signal, panel C. Furthermore, a liver section from a non-tolerized animal that had received a human hepatocyte transplant also produced no stained cells after 6 weeks, panel D. Scanning many fields also showed that most of the positive cells at 6 weeks were in groups, while sections taken at 2 weeks showed scattered single cells, predominantly. Because the injected cell suspensions were predominantly single cells, and because the fluorescence data at 2 weeks showed predominantly isolated single cells, the finding of groups of cells at 6 weeks suggests that the human cells transplanted into tolerized rats not only survived, but proliferated to some extent in the host liver environment.

7. EXAMPLE: INDUCTION OF TOLERANCE TO HUMAN  
HEPATOCYTES BY INTRATHYMIC INJECTION IN  
NEONATAL RATS

Injection of human hepatocytes was performed according to the method  
5 of Fabrega et al., 1995, Transplantation 59:1362-1364.  $10^2$  human hepatocytes in  $5\ \mu\text{l}$   
sterile medium were injected into the thymuses of 1-2 hour old newborn rats. Five  
days following intrathymic injection,  $10^5$  human hepatocytes in  $200\ \mu\text{l}$  sterile medium  
was injected into the spleen (Marucci et al., 1997, Hepatol. 26:1195-1202). Blood  
was collected by tail vein puncture at the time of intrathymic injection and at weekly  
10 intervals following intrasplenic injection of hepatocytes and assayed for human  
albumin by Western blot analysis.

A representative Western blot of serum from one of 6 animals after  
intrathymic tolerization, followed by transplantation of human hepatocytes, is shown  
in FIGURE 7. Lane 1 contains 10 ng standard human albumin; lane 2 contains 10 ng  
15 standard rat albumin and lanes 3-7 contain sera taken at the indicated times after  
transplantation of human hepatocytes. The data show that human albumin production  
increased until about 5 weeks and then remained stable. The other five animals had  
shown similar results.

8. EXAMPLE: A MODEL FOR HEPATITIS B VIRUS INFECTION

20 8.1 PURIFICATION OF HBV PARTICLES FROM HEPG2 2.2.15 CELLS

To test the possibility of infection of human hepatocytes *in vivo*,  
infectious HBV particles were prepared from the HepG2 2.2.15 cell line (obtained  
from Dr. George Acs, Mt. Sinai School of Medicine, NY) which contains an  
integrated tandem repeat genome of HBV ayw strain. The cell line actively secretes  
25 infectious virus into the medium (Sells et al., 1988, J. Virol 62:2836-2844). Culture  
medium from HepG2 2.2.15 was clarified by centrifugation at 5,000 rpm,  $4^\circ\text{C}$  for 30  
min. The supernatant was layered on a 5 ml 25% sucrose cushion in TEN buffer (150  
mM NaCl, 20 mM Tris-HCl, pH 7.4) and centrifuged at 25,000 rpm for 16 hrs at  $4^\circ\text{C}$ .  
The resulting pellet was resuspended in TEN buffer, applied onto a 20-50%  
30 continuous CsCl gradient, and centrifuged at 35,000 rpm for 16 hrs at  $4^\circ\text{C}$ . Fractions

with buoyant densities between 1.24 g/ml and 1.28 g/ml containing HBV virus were collected, dialyzed against TEN and sterile filtered through 0.22  $\mu$  filters.

## 8.2 INFECTION OF HUMAN HEPATOCYTES TRANSPLANTED INTO TOLERIZED RAT HOSTS

5 To determine whether human hepatocytes in rat liver could be infected with human hepatitis virus, one week after human hepatocyte transplantations, tolerized rats were given intrasplenic injections of  $10^4$  hepatitis B viral particles (purified from HepG2 2.2.15 cells as described above) in 50  $\mu$ l. Control animals tolerized but without human hepatocyte transplants, and tolerized transplanted animals  
10 without HBV were used as controls. Liver sections were removed by liver biopsy at 1 week, and partial hepatectomy at 6 weeks and 14 weeks post HBV treatment and analyzed as described below.

## 8.3 IDENTIFICATION OF THE PRESENCE OF HUMAN LIVER CELLS AND HBV INFECTION

15 To identify and quantify human hepatocytes in host liver, immunohistochemical staining for human albumin was used. Visualization of cells infected with HBV was similarly achieved by immunohistochemical staining for Hepatitis B Surface Antigen (HBsAg). Liver tissues were flash frozen in liquid nitrogen immediately following removal. Samples were prepared according to the  
20 method of Osborne and Weber (1982, Meth. Cell Biol. 24:97-132) with minor modifications. Liver cryosections 6  $\mu$ m thick were fixed in 4% paraformaldehyde for 15 min at 25°C, and washed 3 times with phosphate buffered saline (PBS) pH 7.2. Liver sections were quenched with 10% non-fat milk in PBS for 30 min at 25°C followed by successive incubations with 1/1000 dilution of rabbit anti-human albumin  
25 (Sigma). Identical sections were stained with 1/1000 dilution of goat anti-HBV surface antigen (DAKKO). Each section was incubated for 2 hrs at room temperature. Between each primary antibody incubation, liver sections were exposed to 10% non-fat milk in PBS containing .05% NP-40 and 3 times with 10% non-fat milk in PBS alone. Texas red-conjugated anti-rabbit antibody (1/100 dilution) was used to develop

anti-human albumin and FITC conjugated anti-goat (1/100 dilution) antibody was used to develop anti-HBsAg. Sections were incubated with the second antibodies for 30 min at 25°C. Following 3 washes with 10% non-fat milk in PBS, and a final PBS wash, sections were treated with anti-fade 2.5% 1/4-diazobicyclo-[2.2.2]-octane (DABCO) (Sigma), covered with cover slips and stored at 20°C in light-proof boxes. Immunofluorescence of liver sections were visualized with Zeiss Scanning laser confocal microscope (Model LSM-410, Carl Zeiss, New York) using 63X objective. Frozen sections of chimeric liver were incubated with antibodies against human albumin and HBV surface antigen to detect the presence of human albumin and HBV proteins. Human albumin was detected with Texas red conjugated secondary antibody and HBV surface antigen detected with the use of FITC-conjugated second antibody. FIGURE 8A-F clearly demonstrates that both human albumin, left panels 8A, 8C and 8E, and HBV surface antigen, right FIGURES 8B, 8D, and 8F, were clearly detectable within cells in livers of tolerized rats transplanted with human hepatocytes followed by HBV treatment at 1 week (FIGURES 8A and 8B); 6 weeks (FIGURES 8C and 8D); and 14 weeks (FIGURES 8E and 8F). Furthermore, the staining for HBsAg appears to present only in cells that also stained for albumin. However, many cells that contained albumin were also positive for HBV. In a liver sample obtained 14 weeks after injection with HBV, FIGURE 9A-H shows that albumin stained cells were only found in animals that had been tolerized and had received transplanted human hepatocytes (FIGURES 9A and 9C). Animals without human cell transplants that received HBV had no albumin signal (FIGURE 9E). Only tolerized animals that received human hepatocyte cell transplants prior to infection had HBsAg staining (FIGURE 9B). As expected, the same animals without HBV infection failed to show any HBsAg staining indicating that the signal seen in FIGURE 9B was not a non-specific artifact. Furthermore, animals that had no human cells, but did receive HBV injection also had no HBsAg signal (FIGURES 9E and 9F). The data indicate that injected virus had already been completely cleared by the time the liver sample was obtained and that the signal observed in row 1 was due to the infection of cells, and was not an artifact of circulating injected HBV. FIGURES 9G and 9H show that, in the same samples as depicted in FIGURES 9A and 9B, but without primary antibody,

there was no signal corresponding to either albumin (FIGURE 9G) or HBsAg (FIGURE 9H), indicating the staining was not due to non-specific binding of second antibody to the tissue specimens.

#### 8.4 HISTOLOGICAL EVALUATION OF LIVERS EXPOSED TO HBV

5 To determine whether exposure of human hepatocyte transplants to HBV could result in a histological hepatitis *in vivo*, serial slide sections were stained with hematoxylin and eosin and examined in a blinded fashion by a pathologist. The results, discussed below, are shown in FIGURES 13A-C and 14A-C.

#### 8.5 ASSESSMENT OF FUNCTION OF TRANSPLANTED

##### 10 HUMAN HEPATOCYTES

Because albumin synthesis is a selective function of hepatocytes, levels of albumin mRNA were used to determine the activity of transplanted human cells in host liver. To accomplish this, specific primers for human and rat (control) albumin were used. HBV mRNA was detected similarly. Total RNA was extracted from 100  
15 mg liver tissue with acid guanidinium thiocyanate according to the method of Chomczynski and Sacci (1987, Anal. Biochem. 162: 156-159). Poly A + RNA was isolated from total RNA by the method of Aviv and Leder (1972, Proc. Natl. Acad. Sci. USA 69: 1408-1412). RNA was reverse transcribed and amplified by polymerase chain reaction according to the method of Berchtold (1989, Nucl. Acids Res. 17: 453)  
20 with some modifications. Briefly, 10  $\mu$ g total RNA or 1  $\mu$ g polyA + RNA was mixed with 2 pmol of random primer (Gibco/BRL, Gaithersburg, MD) at 70°C for 15 min, and then cooled on ice. Two hundred units of Moloney Murine Leukemia Virus reverse transcriptase (Gibco/BRL, Gaithersburg, MD) was used to reverse transcribe the RNA for 50 min at 42°C. Reaction was stopped by heating to 70°C for 15 min,  
25 after which the cocktail was chilled on ice and treated with 10  $\mu$ g RNase A at 37°C for 20min.

From the total cDNA, polymerase chain amplification of human albumin was performed using, as antisense primer, 5'- CCTTGGTGTGATTGCCTTTGCTC-3' (SEQ ID NO: 4) and as sense primer,



5' -CATCACATCAACCTCTGTCTGACC-3' (SEQ ID NO: 5). If present, the albumin cDNA would generate a characteristic 315 bp fragment of the human albumin gene spanning nucleotides 176-491. For rat albumin, an antisense primer 5' - ATAGTGTCCCAGAAAGCTGGTAGGG-3' (SEQ ID No: 6) and a sense primer: 5' - CGGTTTAAGGACTTAGGAGAACAGC-3' (SEQ ID No: 7) were used to generate an expected 400 bp fragment of the rat albumin gene spanning nucleotides 104-504. To search for the presence of HBV in liver, an antisense primer 5' - ATCTTCTGCGACGCGGCGATGGAGATC-3' (SEQ ID No: 8) and a sense primer 5' - CTCTGCTGGGGGGAATTGATGACTCTAGC-3' (SEQ ID NO: 9) were used to generate a characteristic 355 bp fragment of the ayw HBV genome spanning nucleotides 2079-2434. One third of the total cDNA was mixed with 100 pmol of amplification primers and 2.5 U Taq polymerase and amplified at 1 cycle at 94°C for 3 min, then for 38 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and then 1 cycle at 72° for 5 min. The PCR products were analyzed on 1.0% agarose gels in Tris-Borate-Acetate buffer.

FIGURE 10, lanes 1 and 2 show that the RT-PCR products of albumin from rat and human can be completely distinguished from each other based on electrophoretic mobility. Lane 4 shows RNA from HepG2 2.2.15 cells demonstrating a strong level of albumin synthesis in these cells. In tolerized animals that had human hepatocyte transplants, the rat albumin signal had the same intensity in cells infected with HBV (lanes 5 and 6) compared to those which were not (lanes 7 and 8). However, the human albumin mRNA signal in cells infected with HBV (lanes 5 and 6) appeared to be increased compared to non-infected cells (lanes 7 and 8). As expected, control animals that had no human hepatocyte transplants, but were administered HBV, had no detectable human albumin signal (lanes 9 and 10).

A time course of the levels of human albumin and HBV message is shown in Fig. 11. Compared to albumin RNA from human liver cells in the upper panel, lane 3, and from HepG2 2.2.15 cells lane 4, human albumin messenger RNA signal at 387 bp was easily detectable at one week after HBV infection, lane 5; with at least equal signal from week 6 and 14, lanes 6 and 7, respectively.

FIGURE 11, bottom panel shows that HBV RNA could be detected in livers by the presence of a 355 bp band at 1 week after injection, lane 5. The intensity appeared to increase slightly at 6 weeks, lane 6; and remained strong at 14 weeks, lane 7, after HBV inoculation. The same primers were used to amplify HBV RNA from a human liver cell line HepG2 2.2.15 that continually produces HBV and was used as the source of HBV viral particles for the infections. FIGURE 12 shows that HBV RNA could not be detected in livers of tolerized rats that did not receive hepatocyte transplantation, but received HBV (lanes 9, 10), indicating that the signal was not due to residual injected HBV.

FIGURE 13A-C depicts slides of livers from tolerized rats, transplanted with human hepatocytes and infected with HBV, at low power (20X). FIGURE 13A shows that liver, one week after infection, has normal architecture for that stage in life and no evidence of inflammatory cell infiltration. However, after 6 weeks (FIGURE 13B), foci of necrosis and mononuclear cell infiltrates can be seen. FIGURE 13C shows substantial mononuclear inflammation after 14 weeks with an increase in Kupffer cells as well. FIGURE 14A-C shows that at high power (40X), the infiltrates are more easily seen to be to be mononuclear cells surrounding areas of necrosis at 6 weeks (FIGURE 14B). At 14 weeks, the inflammation extends into the surrounding parenchyma (FIGURE 14C).

#### 8.6. DETECTION AND QUANTITATION OF HBSAG IN RAT SERUM

To follow the course of infection, levels of HBsAg in rat serum were measured using an EIA kit for HBV surface antigen (Abbott Labs, Abbott Park, IL) according to the manufacturer's protocol. Briefly, 10  $\mu$ l serum in 190  $\mu$ l saline was mixed with anti-HBs (mouse) monoclonal antibody coated beads and 50  $\mu$ l of horseradish peroxidase conjugated anti-mouse secondary antibody and incubated at room temperature for 16 hours. Then the incubation solution was removed and the beads were washed six times with 10 ml distilled water, and the beads were transferred to clean assay tubes and incubated with 300  $\mu$ l of freshly prepared o-phenylenediamine substrate and quantitated using a spectrophotometer at 492 nm.

Assays were done in triplicate and the results (see Tables 1, 2 and 3, third column)

were expressed as means  $\pm$  S.D. in units of pg/ml serum.

#### 8.7. DETECTION OF SERUM ALANINE AMINOTRANSFERASE (ALT)

To determine whether HBV infection was associated with any liver damage, serum was collected from rats as a function of time after injection, and serum ALT values determined in triplicate from 10  $\mu$ l serum using a commercial ALT detection kit (Sigma). All assays were done in triplicate and results are expressed as means  $\pm$  S.D. International Units (IU)/ml.

Group 1 animals were treated by (i) intrafetal injections with human hepatocyte lysates into the peritoneums at 17 days post-conception; (ii) intrasplenic saline injection at birth; and (iii) one week later, purified HBV harvested from a human hepatoma cell line was administered by intrasplenic injection.

TABLE 1

| Time Post-HBV Injection (days) | ALT (IU/L)  | HBsAg (pg/ml)  |
|--------------------------------|-------------|----------------|
| 0                              | 28 $\pm$ 15 | Not detectable |
| 1                              | 44 $\pm$ 20 | Not detectable |
| 8                              | 22 $\pm$ 12 | Not detectable |
| 10                             | 25 $\pm$ 10 | Not detectable |

As shown in Table 1, animals that received no tolerization or human hepatocytes, but were injected with HBV had no significant changes in ALT as a measure of liver cell damage, or detectable HBsAg in the serum even as soon 1 day after injection of HBV through at least 10 days. The data confirm that HBV does not cause hepatic damage in rats and that the virus is rapidly cleared from the circulation.

Animals in Group 2 were treated by (i) intrafetal injection with human hepatocyte lysate into the peritoneums at 17 days post-conception; (ii) intrasplenic injection of 2 million human primary hepatocytes at birth; and (iii) 1 week post hepatocyte transplantation, saline was injected intrasplenically.

TABLE 2

| Time Post-HBV Injection (days) | ALT (IU/L) | HBsAg (pg/ml)  |
|--------------------------------|------------|----------------|
| 0                              | 25 ± 15    | Not detectable |
| 1                              | 22 ± 20    | Not detectable |
| 8                              | 30 ± 5     | Not detectable |
| 10                             | 25 ± 16    | Not detectable |

As shown in Table 2, animals tolerized with human hepatocytes that received human hepatocyte transplants, but no HBV, also had normal ALT and undetectable HBsAg throughout the 10 days. Thus, without inoculation with HBV, there was no serological evidence of hepatotoxicity or circulating HBV.

Animals in Group 3 were treated by (i) transuterine injection into the peritoneum at 17 days post-conception with primary human hepatocyte lysate; (ii) intrasplenic injection of 2 million human primary hepatocytes at birth; and (iii) HBV was injected intrasplenically at 1 week post-hepatocyte transplantation.

TABLE 3

| Time Post-HBV Injection (days) | ALT (IU/L) | HBsAg (pg/ml)  |
|--------------------------------|------------|----------------|
| 0                              | 22 ± 15    | Not detectable |
| 1                              | 47 ± 10    | 0.2 ± .01      |
| 4                              | 86 ± 15    | 0.1 ± .05      |
| 8                              | 132 ± 15   | 0.4 ± 0.1      |
| 10                             | 179 ± 20   | 0.5 ± .15      |

As shown in Table 3, in this group which was tolerized and received human hepatocytes and HBV, ALT levels were normal until day 4 when the level doubled to 86 IU/L. By day 10, the ALT had doubled again to 179 IU/L. The HBsAg was detectable at 0.1-0.2 pg/ml through day 4. However, the levels doubled to 0.4 and reached 0.5 pg/ml day 10. These data suggest that viral antigen and likely viral levels increase early in the process, and are followed by liver cell damage. This is supportive of an inflammatory process triggered by the injection of HBV, but only in animals that have human hepatocytes.

9. EXAMPLE: FURTHER STUDIES DEMONSTRATING  
THAT HUMAN HEPATOCYTES TRANSPLANTED INTO  
GENETICALLY IMMUNOCOMPETENT RATS ARE  
SUSCEPTIBLE TO INFECTION BY HEPATITIS B VIRUS  
IN SITU

9.1 MATERIALS AND METHODS

9.1.1 ANIMALS

Sprague-Dawley female rats, 15 days pregnant, were obtained from Charles River Co. Inc. (Wilmington, MA), maintained on 12 hr light-dark cycles, and fed *ad lib* with standard rat chow. All animal procedures were approved by Institutional Animal Care and Use Committee and conformed to USDA and NIH animal usage guidelines.

9.1.2 CELLS

Primary human hepatocytes were obtained from Clonetics Corp. (Walkersville, MD) and kept frozen at -70 °C. For use in experiments, frozen cells were thawed at 37 °C, washed with Hepatocyte Culture Medium (Clonetics Corp) containing 5 mg/ml insulin and 0.39 µg/ml dexamethasone (SingleQuote, Clonetics Corp) and spun at 50 x g, 10 min, 4 °C. Microscopic examination of the recovered cells revealed a viability of 65% as determined by trypan blue exclusion, and parenchymal hepatocyte composition of greater than 90%. For injections, cells were suspended in 500 µl phosphate buffered saline (PBS), and used immediately. HepG2 2.2.15 human hepatoblastoma cells that constitutively produce infectious HBV particles (Sells et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84: 1005-1009) were maintained in DMEM containing 10% FBS as described previously (Sells et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84: 1005-1009).

9.1.3. INDUCTION OF IMMUNE TOLERANCE  
TO HUMAN HEPATOCYTES

The abdominal cavities of pregnant rats were exposed by laparotomy under sterile conditions, and the gravid uteri trans-illuminated. Primary human  
5 hepatocytes,  $10^5$  cells in 10  $\mu$ l PBS, were injected with a Hamilton syringe, using sterile technique through the uterine wall into the peritoneal cavities of fetal rats 17 days post-conception, under a dissecting microscope. Control animals were treated identically except that they were injected with saline instead of cells.

9.1.4 MIXED LYMPHOCYTE ASSAYS

10 To assess immune tolerance, mixed lymphocyte assays were performed according to the method of Schwartz *et al.* (1975, J. Immunol. 115: 1330-1338). In these assays, responder spleen cells were stimulated to proliferate by exposure to stimulator cells if they are not tolerant to those cells. Two weeks after birth, spleens from rats that had intrafetal injections of human hepatocytes were removed, and  
15 aliquots of cells prepared as responder cells. Stimulator cells consisting of  $3 \times 10^5$ /0.5 ml human hepatocytes gamma irradiated with 2000 R to inhibit proliferation, were mixed with  $1.0 \times 10^6$  responder spleen cells/0.5 ml from tolerized and saline-treated control animals. An aliquot of spleen cells from normal untreated rats, as well as irradiated hepatocytes alone, mixed with an equal volume of buffer were used to  
20 measure background proliferation under identical conditions. Cell suspensions were pulsed with 1  $\mu$ Ci of [ $^3$ H]-thymidine [80.0 Ci/mmol, Amersham Life Science] and then incubated for 72 hrs at 37 ° C, under 10% CO<sub>2</sub>, in RPMI medium containing 5% fetal bovine serum. Cells were then harvested onto Whatman glass fiber filters, washed once with cold PBS pH 7.0, then cold 10% trichloroacetic acid, and finally  
25 cold 95% ethanol. The radioactivity retained by the filters was measured in a scintillation counter. All experiments were performed in triplicate, and thymidine incorporation results expressed as means  $\pm$  S.D. in units of cpm/ $10^6$  cells.

#### 9.1.5. TRANSPLANTATION OF HUMAN HEPATOCYTES

Within 24 hrs after birth, groups of tolerized neonatal rats were transplanted with human hepatocytes by injection into the spleens according to the method of Marucci et al. (1997), Hepatology 26: 1195-1202). Incisions were made at the left paracostal areas, the spleens visualized and  $2 \times 10^6$  human primary hepatocytes in 100  $\mu$ l sterile medium were injected slowly into the organ.

#### 9.1.6 PURIFICATION OF HBV FROM HEPG2 2.2.15 CELLS

Infectious HBV particles were harvested from the medium of HepG2 2.2.15 cells similar to the method described in Liang et al., 1993, J. Clin. Invest. 91: 1241-1246. In brief, culture medium from HepG2 2.2.15 cells was centrifuged at 5000 rpm (4000 x g) at 4 °C for 30 min. The clarified medium was layered on a 25% sucrose cushion in Tris-EDTA-NaCl (TEN) buffer, and centrifuged at 25,000 rpm (100,000 x g) for 16 hrs at 4°C. The pellet was resuspended in TEN buffer and applied onto 20-50% continuous cesium chloride gradient at 35,000 rpm for 16 hrs at 4°C. Fractions with buoyant densities between 1.24 g/ml and 1.28 g/ml contained HBV virus, and were collected, dialyzed against TEN, sterile filtered through 0.22  $\mu$  filters before injection. The amount of virus present was determined by dot blots using an DNA fragment corresponding to nt 0-1802 of the HBV genome, as a probe, and quantitated by using serial dilutions of full length HBV DNA excised from padwR9 (Liang et al., 1993, J. Clin. Invest. 91: 1241-1246) to generate standard curves.

#### 9.1.7 HBV INOCULATION OF RATS

One week following human hepatocyte transplantation, groups of tolerized rats were inoculated with  $10^5$  HBV particles in 100  $\mu$ l TEN buffer by intrasplenic injection. Controls consisted of tolerized neonatal rats from the same litter that did not receive human hepatocyte transplantation, but were also given identical HBV inoculations, as well as tolerized rats from the same litter that received saline instead of human hepatocytes, but no HBV.

#### 9.1.8 VISUALIZATION OF HUMAN LIVER CELLS AND HBV INFECTION

Immunohistochemical analysis for the presence of human albumin and hepatitis B virus surface antigen (HBsAg) were performed according to the method of Osborne and Weber (1982, Meth. Cell Biol. 24: 97-132) with minor modifications. Liver tissues were flash frozen in liquid nitrogen immediately following removal from animals, and 5  $\mu$  thick liver cryosections were fixed in 4% paraformaldehyde for 15 min at 25°C, washed several times with PBS pH 7.2. Liver sections were quenched with blotto (10% non-fat milk in PBS) for 30 min at 25°C followed by successive or single incubations with 1/1000 dilution of anti-human albumin (Sigma) and 1/1000 anti-HBV surface antigen (Dakko) for 2 hrs at room temperature. Between each primary antibody incubation, liver sections were washed with blotto containing 0.5% Tween-20. Texas Red-conjugated anti-rabbit antibody at 1/1000 dilution (Amersham Life Science) was used to develop anti-human albumin, and FITC-anti-mouse antibody at 1/1000 dilution (Boehringer Mannheim) for anti-HBsAg. Sections were incubated with the second antibodies for 30 min at 25°C and treated with antifade 2.5% 1/4-diazobicyclo-[2.2.2]-octane (DABCO) (Sigma), covered with cover slips and stored at -20°C in light proof boxes. Immunofluorescence of liver sections was analyzed using a Zeiss Scanning laser confocal microscope (Model LSM-410) using 63X objective.

#### 9.1.9 INSITU HYBRIDIZATION

To identify cells containing human albumin, and HBV DNA in transplanted human hepatocytes, in situ hybridization was performed according to the method of Pardue (1985, in *Nucleic Acid Hybridization: A Practical Approach*, eds. B.D. Hames, B.D. and Higgins, S.E. (IRL Press, Oxford), pp. 179-202). Briefly, 5  $\mu$  frozen liver sections were fixed with 4% paraformaldehyde, washed with PBS and digested with pronase. Sections were incubated with a [<sup>32</sup>P]-labeled 355 bp fragment of HBV cDNA labeled with specific activity of  $4 \times 10^8$  cpm/ $\mu$ g. Sections were hybridized for 4 hrs at 37°C. Following hybridization and washing, sections were exposed to Kodak film emulsion for one week, developed, and counterstained with



toluidine blue. Radioactivity of the sections were visualized by dark field illumination using a Leitz Labor Lux S microscope using 40X objective. Tolerized rats without transplantation and inoculated with HBV, and untreated rats served as controls.

#### 9.1.10 DETECTION OF HBV DNA IN RAT SERUM

5 After inoculation with HBV, serum samples were collected weekly. To detect HBV DNA, DNA was extracted from 30  $\mu$ l of rat serum, incubated with 100  $\mu$ g/ml proteinase K in .05 M Tris-HCl, pH 8.0, 0.1M EDTA and 0.5% SDS, overnight at 55°C. DNA was purified by phenol-chloroform (Gross-Bellard et al., 1973, Eur. J. Biochem. 36: 32-38).

10 An antisense primer 5'-ATCTTCTGCGACGCGGCGATGGAGATC-3' (SEQ ID NO:8) and a sense primer 5'-CTCTGCTGGGGGGAATTGATGACTCTAGC-3' (SEQ ID NO:9) were used to generate an expected 355 bp fragment of adw HBV genome spanning nt 2079-2434. One third of total cDNA was mixed with 100 pmol of amplification primers and 2.5 U  
15 Taq polymerase, and amplified at 1 cycle 94 °C for 3 min; 38 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 minute; and the final elongation reaction at 72 °C for 5 minutes. The PCR products were analyzed on 1.0% agarose gels in Tris-Borate-Acetate buffer and visualized by ethidium bromide staining.

#### 9.1.11 QUANTITATION OF HBV IN RAT LIVER AND SERUM

20 To measure the HBV viral load in rat serum, DNA was extracted from 30  $\mu$ l of rat serum using the method mentioned above and fixed onto nylon membranes (Amersham). The amount of HBV viral DNA was quantitated by DNA dot-blot hybridization (Scotto et al., 1983, Hepatology 3 : 279-284). Serial dilutions of standard adw HBV DNA from 1 million copies to one thousand copies were used to  
25 determine the number of HBV viral particles/ml serum. Blots were hybridized overnight at 60°C to [<sup>32</sup>P]-dATP-labeled full length HBV DNA probe excised from padwR9 plasmid (Liang et al., 1993, J. Clin. Invest. 91: 1241-1246). Following hybridization and washing, filters were visualized by exposure to XAR film (Kodak).

#### 9.1.12 DETECTION AND QUANTITATION OF HBsAg IN SERUM

Levels of HBsAg in rat serum were measured as a function of time after inoculation using an enzyme-linked immunoassay kit for HBV surface antigen (Abbott Labs) according to the manufacturer's protocol. Ten  $\mu$ l serum in 190  $\mu$ l  
5 saline was mixed with anti-HBsAg (mouse) monoclonal antibody-coated beads and 50  $\mu$ l of horse radish peroxidase-conjugated anti-mouse secondary antibody and incubated at 25°C for 16 hrs. The incubation solution was removed and the beads washed with 10 ml distilled water, and then incubated with 300  $\mu$ l of o-phenylenediamine substrate. Antigen was quantitated using a spectrophotometer at  
10 492 nm. Assays were done in triplicate, and the results expressed as means  $\pm$  S.D. in units of pg/ml serum.

#### 9.1.13 ASSAYS FOR REPLICATIVE HBV DNA

To determine whether inoculated HBV produced progeny virus, covalently closed circular DNA was assayed by PCR using primers that amplify a  
15 region that spans the sequences of the plus strand that are incomplete in viral particles, but are converted to complete covalently closed circles upon infection. DNA was extracted from 20 mg of liver according to the method of Arrigo et al. (1989, J. Virol.  
63: 4875-4881). The primers used were: sense  
GCCGGTCTGGAGCAAAGCTCATCGG (SEQ ID NO:10) spanning nt 1306-1380  
20 and antisense GGCGGTGTCTAGGAGATCTCTGAC (SEQ ID NO:11) spanning nt 1981-2004 of adwHBV genome. After amplification, an anticipated 698 bp band was sought by agarose gel electrophoresis in the presence of ethidium bromide.

### 9.2 RESULTS

25 FIGURE 15, lane 3 shows that spleen cells from rats that had not been injected intrafетally with hepatocytes, were stimulated to proliferate, incorporating  $6.0 \times 10^4 \pm 6 \times 10^3$  cpm [ $^3$ H]-thymidine/ $10^6$  cells when exposed to (irradiated) human hepatocytes. In contrast, exposure of spleen cells from animals that were tolerized intrafетally with human hepatocytes, lane 4, resulted in a significantly [ $p < .002$ ] lower  
30 uptakes,  $2.9 \times 10^4 \pm 10^3$  cpm/ $10^6$  cells. This uptake was not significantly different

from a background of spleen cells alone from rats injected intrafetally with only saline, in the absence of any stimulator cells, lane 1. Lane 5 shows that intrafetal hepatocyte injections alone, without subsequent hepatocyte transplantations were sufficient to induce tolerance as evidenced by a lack of a significant increase in proliferation in the presence of human hepatocytes. Finally, lane 2 shows that irradiated hepatocytes alone without spleen responder cells had no significant radioactive incorporation indicating that the vast majority of the observed uptake of radioactivity in mixtures of the two cell types was due to spleen cells. The data confirm that the dose of irradiation was sufficient to block significant hepatocyte proliferation.

To visualize human hepatocytes, cells producing human albumin were detected immunohistochemically with a Texas Red-conjugated second antibody. Similarly, cells containing HBsAg were detected with a FITC-second antibody in frozen sections of liver. FIGURE 16A-F shows that in representative liver sections taken 15 weeks after HBV inoculation, human albumin, FIGURE 16A, and HBV surface antigen, FIGURE 16B (serial sections) can be detected in cells with brightly stained cytoplasm. Cells positive for albumin were not uniformly distributed throughout the liver lobule, but appeared to be concentrated in groups of several cells each, interspersed between other areas that had few positive cells. Some of the cells, approximately 30%, stained positive for both albumin and for HBsAg. However, no cells that were positive for HBsAg, were completely negative for human albumin. As expected, livers from tolerized rats transplanted with human hepatocytes, but not inoculated with HBV were positive for human albumin only, FIGURE 16C, and negative for HBV surface antigen, FIGURE 16D. Control animals that received intrafetal tolerization, and not human hepatocyte transplantation, but were inoculated with HBV, were negative for both human albumin, FIGURE 16E, and HBV surface antigen, FIGURE 16F, indicating that the observed positive staining in FIGURES 16A and 16B was not due to non-specific or cross-reactions against rat albumin, other endogenous antigens, or injected HBV antigens.

It is possible that the positive staining of cells detected in FIGURE 16A-F was due to uptake of antigen, but not HBV DNA. To determine whether that

was the case, cells containing HBV DNA were sought by in situ hybridization.

FIGURE 17C shows that liver sections from rats that were tolerized, transplanted with human hepatocytes, and inoculated with HBV had multiple positive cells as evidenced by white spots on the negative (arrows). In contrast, control livers from rats tolerized, transplanted with human hepatocytes, but not inoculated with HBV, FIGURE 17A, had no signal, indicating that the observed positive cells in FIGURE 17C were not simply due to an artifact present in human cells. Similarly, rats that were tolerized, and not transplanted, but did receive HBV, FIGURE 17B, also had no signal. Hence, the signal was not a residue of input HBV. Finally, FIGURE 17D shows that, as expected, livers from rats that were neither transplanted nor exposed to HBV, failed to demonstrate any hybridization signal.

FIGURE 18A shows that the presence of HBV DNA in serum was confirmed by the finding of an expected 355 bp fragment of the adw HBV genome spanning nt 2079-2434 generated by PCR. For PCR, the same primers were used to amplify HBV DNA from a human liver cell line HepG2 2.2.15 as a positive control, lane 3. The 355 bp fragment of HBV can clearly be detected in serum of two representative rats, lanes 4-6 and 7-9, respectively, both of which were tolerized, had human hepatocyte transplantation and HBV inoculation. Serum HBV DNA was detectable at 1 week after infection, lanes 4 and 7, and remained detectable through 15 weeks after inoculation, lanes 6 and 9. HBV DNA was not detected in serum from tolerized rats with human hepatocyte transplantation alone (with no exposure to HBV), lanes 10 and 11, in serum of two representative tolerized rats that did not receive human hepatocytes, but were exposed HBV, lanes 12-14 and 15-17, at 1, 5 and 15 weeks after HBV inoculation. FIGURE 18B shows that serum HBV DNA quantitated by dot-blots from two representative rats transplanted with human hepatocytes had a mean value of 5000 copies/ml at 1 week, dots A2 and B2; 10,000 copies/ml at 5 weeks, dots A3 and B3; and increased to a mean of 50,000 copies/ml by 15 weeks after exposure, dots A4 and B4. In contrast, serum from tolerized animals with hepatocytes alone, dots C1 and D1, and serum from tolerized animals infected with HBV alone, dots C2-4 and D2-4, showed no detectable HBV DNA at similar

time points. HBV DNA was not found in serum of control untreated rats, dots A1 and B1.

To determine whether HBV replication could occur in human hepatocytes transplanted in rat livers, covalently closed circular HBV DNA was assayed using PCR primers specific to region of HBV genome that is incomplete in the plus strand of the viral particle, but is covalently closed upon HBV replication. FIGURE 19A-B shows that PCR detected a 698 bp band, corresponding to the expected size of nt 1306-2004 of a covalently closed circular adw HBV genome, in the livers, FIGURE 19A lanes 4-5, and sera, FIGURE 19B lanes 3-4, respectively, of two representative rats that were transplanted with human hepatocytes and infected with HBV, at 15 weeks after HBV inoculation. The same band was not detected in rats transplanted with human hepatocytes alone, and not exposed to HBV, in liver (FIGURE 19A lane 6) or serum (FIGURE 19B lane 5). No signal was found in tolerized rats not transplanted, but exposed to HBV, in liver (FIGURE 19A lanes 7-8) or serum (FIGURE 19B lane 6), indicating that the generated PCR fragment was not a non-specific product arising from the tolerization procedure, or the presence of residual input HBV, or non-specific amplification of a host sequence.

FIGURE 20 shows that HBsAg was detectable in serum of transplanted and HBV inoculated rats, solid circles, by 24 hrs after inoculation, and increased to 0.75 pg/ml where levels remained relatively constant through the period of observation of 60 days. Control rats that were transplanted with human hepatocytes alone, and not exposed to HBV, open squares, or exposed to HBV in the absence of transplanted human hepatocytes, crosses, had no detectable antigen at any time point.

### 9.3 DISCUSSION

Human hepatocytes previously infected with human viruses have been introduced into immunodeficient animals in the past. For example, hepatitis C virus-infected human liver has been transplanted into animals with ablated marrow reconstituted with SCID marrow (Galun et al., 1995, J. Infect. Dis. 172: 25-30). In another example, mice with ablated bone marrow, reconstituted with SCID bone marrow were transplanted with an HBV-infected human liver fragment under the

kidney capsule (Ilan et al., 1999, *Hepatology* 29: 553-562). Because the liver specimen was transplanted en bloc, some human liver matrix and non-parenchymal cells accompanied the hepatocytes in the fragment. Therefore, some of the hepatocytes would have retained natural relationships to extracellular matrix and other human cells. In addition, the specimen was pre-infected with HBV. It is, therefore, not surprising that HBV viremia was found in the host following the transplantation. Brown et al. recently transplanted into RAG-2-deficient mice, immortalized human hepatocytes permanently transfected with an HBV genome and showed long term viremia in another example of an immunodeficient model (Brown et al., 2000, *Hepatology* 31: 173-181). In the experiments described herein, uninfected normal hepatocytes were separated from matrix, and as well as other human cells during the isolation procedure, and therefore, were transplanted into a completely unnatural environment. Nevertheless, the cells maintained a degree of function as evidenced by production of human albumin detected by immunohistochemistry. This is of interest because primary cultured hepatocytes are notorious for the rapidity with which they lose differentiated function and, indeed, are difficult to infect with HBV *in vitro*. The fact that the transplanted hepatocytes were susceptible to HBV infection suggests that the rodent liver environment, although foreign, is still sufficient for retention of this sensitive and particular property of human hepatocytes. The retention of function was also noted in a closely related study by Petersen et al. who transplanted woodchuck hepatocytes into a urokinase RAG-2 mouse and showed that the transplanted woodchuck cells could be infected by inoculation with WHV (Petersen et al., 1998, *Proc. Natl. Acad. Sci. U.S.A.* 95:310-315).

Because of the time frame involved in the above experiments, it is unclear whether the rat inoculations resulted in transient or chronic infections. The rat immune system does not become mature until approximately three weeks after birth. Thus, inoculation with HBV within 24 hrs of birth described here simulates perinatal infection in man. Human perinatal infections are common in regions of the world highly endemic with HBV, and in these cases, it has been observed that the likelihood of progression to chronicity is extremely high presumably due to immaturity of the immune system at the time of infection (Milich et al., 1995, *Springer Seminars in*

Immunopathology 17 :149-166). In addition, a mismatch exists between the human MHC and the rat immune system which could hamper clearance of virus and infected cells. These factors could increase the likelihood of development of chronic infection.

Injection of hepatocytes into the spleen was used as for convenient  
5 access to the liver. It has been shown previously the vast majority of hepatocytes so injected, migrate into the liver (Vroemen et al., 1986, Transplantation 42: 130-135), and exit the sinusoids to enter the host parenchyma (Parker-Ponder et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88 :1217-1221). While the data provided herein indicate that numerous human hepatocytes were present in the host liver after transplantation, and  
10 that some of these cells also had HBV DNA and HBsAg after inoculation of the rats with HBV, the possibility of HBV infection of some of the human hepatocytes that remained in the spleen cannot be excluded. From previous reports, the number of cells would be expected to be less than 15% of that originally injected (Parker-Ponder et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88 :1217-1221).

15 Tolerization was achieved by transuterine injection of human cells into fetal rat on the 17th day of gestation. This is similar to previous studies on mice, in which injection of foreign cells on the 14<sup>th</sup> to 16<sup>th</sup> day of gestation was found to result in tolerance to subsequent cardiac and skin grafts (Kline et al., 1994, Am. Thorac. Surg. 57 :72-75). However, the pioneering work in mice and chicks *in utero* to attain  
20 acquired tolerance to foreign cells was done by Medawar and co-workers (Billingham et al., 1953, Nature 4379: 603-606). Although the exact mechanism of the tolerance is not exactly clear, clonal deletion of specific thymocytes (Pullen et al., 1988, Nature 335 :796-801; McDuffie et al., 1988, J. Immunol. 141:1840-1847), and the involvement of major histocompatibility complex, H-2 (Laconi et al., 1998, Am. J.  
25 Pathol. 153:319-329) in suppressing the immune response to allogenic cells have been postulated to be involved.

Various publications are cited herein, the contents of which are hereby incorporated by reference in their entireties.

### CLAIMS

1. A non-human animal having a liver that comprises human hepatocytes, wherein the animal has a normal immune system but has been rendered tolerant to human hepatocytes.

5           2. The non-human animal of claim 1, wherein the animal carries a transgene that is directly toxic to hepatocytes of the non-human animal but not specifically toxic to human hepatocytes.

          3. The non-human animal of claim 2, where the transgene is a urokinase gene operably linked to a promoter selectively active in liver cells of the  
10   animal.

          4. The non-human animal of claim 3, where the promoter is the albumin promoter.

          5. The non-human animal of claim 1, wherein the animal carries a transgene that is indirectly toxic to hepatocytes of the non-human animal but not  
15   specifically toxic to human hepatocytes, and wherein the toxic effect is induced by administering an exogenous agent to the animal.

          6. The non-human animal of claim 5, where the transgene is a *Herpes simplex* virus thymidine kinase gene operably linked to a promoter selectively active in liver cells of the animal.

20           7. The non-human animal of claim 6, where the exogenous agent is gancyclovir.

          8. The non-human animal of claim 6, where the promoter is the albumin promoter.

          9. A method of preparing a non-human animal having a liver  
25   comprising human hepatocytes, comprising the steps of: (i) inducing tolerance in a non-human host animal toward hepatocytes from a human donor and (ii) introducing hepatocytes from the human donor into the tolerized animal produced in step (i) such that at least some of the introduced human hepatocytes localize in the liver of the non-human animal.



10. The method of claim 9 where tolerance is induced by intraperitoneal injection of an antigen-containing composition selected from the group consisting of a human cell lysate and intact human cells.

5 11. The method of claim 9 where tolerance is induced by intrathymic injection of an antigen-containing composition selected from the group consisting of a human cell lysate and intact human cells.

12. The method of claim 9 further comprising the step of subjecting the animal to a selection pressure which favors the proliferation of human hepatocytes.

10 13. The method of claim 12 where the selection pressure is maturation of the animal which activates the expression of a hepatotoxic transgene in the hepatocytes of the non-human animal host but not the donor human hepatocytes.

14. The method of claim 12 where the selection pressure is provided by exposing the non-human animal to a toxin, wherein donor human hepatocytes are protected against the toxin by transfection of a transgene into the human hepatocytes,  
15 wherein the transgene protects the donor hepatocytes from the toxic effects of the toxin, and wherein hepatocytes of the non-human animal lack the transgene and are therefore not protected against the toxin.

15. The method of claim 14, wherein the protective transgene encodes a product selected from the group consisting of an antisense RNA and a ribozyme  
20 that functionally inactivates a cytochrome selected from the group consisting of 2E1, 1A2 and 3A4, such that the transgene would prevent activation of acetaminophen in human donor hepatocytes but not hepatocytes of the non-human animal.

16. The method of claim 14, wherein the protective transgene encodes a mutant RNA polymerase II that would render the human donor hepatocytes resistant  
25 to phalloidin.

17. The method of claim 13 where the hepatotoxic transgene is a urokinase gene operably linked to an albumin promoter.

18. The method of claim 12 where the selection pressure is the administration of a compound that is metabolized to a toxic substance in hepatocytes  
30 of the non-human animal host but not the donor human hepatocytes.

19. The method of claim 18 where the non-human animal host carries a *Herpes simplex* virus thymidine kinase gene that is selectively expressed in hepatocytes of the animal and the administered compound is gancyclovir.

20. A method for identifying a toxic effect of a test agent, comprising  
5 administering the test agent to a non-human animal having a liver that comprises human hepatocytes and subsequently evaluating whether changes have occurred in at least one marker of bodily function of the animal.

21. A method for identifying a toxic effect of a test agent, comprising  
10 administering the test agent to a non-human animal having a liver that comprises human hepatocytes and subsequently evaluating whether changes have occurred in the viability of human hepatocytes in the animal.

22. A model system for a human liver disease comprising a non-human animal having a liver that comprises human hepatocytes in which an agent associated with causation of the disease in humans has been introduced.

15 23. The model system of claim 22 where the human liver disease is alcohol-associated liver disease and where the non-human animal has been administered an amount of alcohol effective in producing hepatocellular degenerative changes.

20 24. A model system for a human liver disease caused by an infectious agent, comprising a non-human animal having a liver that comprises human hepatocytes infected by the infectious agent.

25 25. The model system of claim 24, where the infectious agent is hepatitis C virus.

26. The model system of claim 24, where the infectious agent is  
25 hepatitis B virus.

27. The model system of claim 24, where the infectious agent is  
hepatitis A virus.

28. The model system of claim 24, where the infectious agent is  
hepatitis D virus.

30 29. The model system of claim 24, where the infectious agent is Yellow Fever virus.

30. The model system of claim 24, where the infectious agent is hepatitis E virus.

31. The model system of claim 24, where the infectious agent is malaria.

5 32. The model system of claim 24, where the infectious agent is cytomegalovirus.

33. The model system of claim 24, where the infectious agent is Epstein Barr virus.

10 34. A model system for a human liver disease caused by a virus, comprising a non-human animal having a liver that comprises human hepatocytes containing a nucleic acid of the virus, where the nucleic acid is selected from the group consisting of a nucleic acid comprised in the genome of the virus and a nucleic acid transcript encoded by the genome of the virus.

15 35. The model system of claim 34, where the virus is hepatitis C virus.

36. The model system of claim 34, where the virus is hepatitis B virus.

37. The model system of claim 34, where the virus is hepatitis A virus.

38. The model system of claim 34, where the virus is hepatitis D virus.

39. The model system of claim 34, where the virus is Yellow Fever virus.

20 40. The model system of claim 34, where the virus is hepatitis E virus.

41. The model system of claim 34, where the virus is cytomegalovirus.

42. The model system of claim 34, where the virus is Epstein Barr virus.

25 43. A method of treating a human subject having a genetic defect, comprising (i) propagating human hepatocytes that correct the genetic defect in a non-human animal, where the human hepatocytes are histocompatible with the human subject; (ii) collecting hepatocytes from the animal; (iii) separating human hepatocytes from non-human hepatocytes; and (iv) introducing the human hepatocytes into the subject.

44. The method of claim 43, where the human hepatocytes are originally obtained from the subject and then engineered to correct the genetic defect prior to propagation in the non-human animal.

5 45. A method of treating a human subject having a damaged liver, comprising (i) propagating human hepatocytes in a non-human animal, where the human hepatocytes are histocompatible with the human subject; (ii) collecting hepatocytes from the animal; (iii) separating human hepatocytes from non-human hepatocytes; and (iv) introducing the human hepatocytes into the subject.

10 46. The method of claim 45, where the human hepatocytes are originally obtained from the subject.

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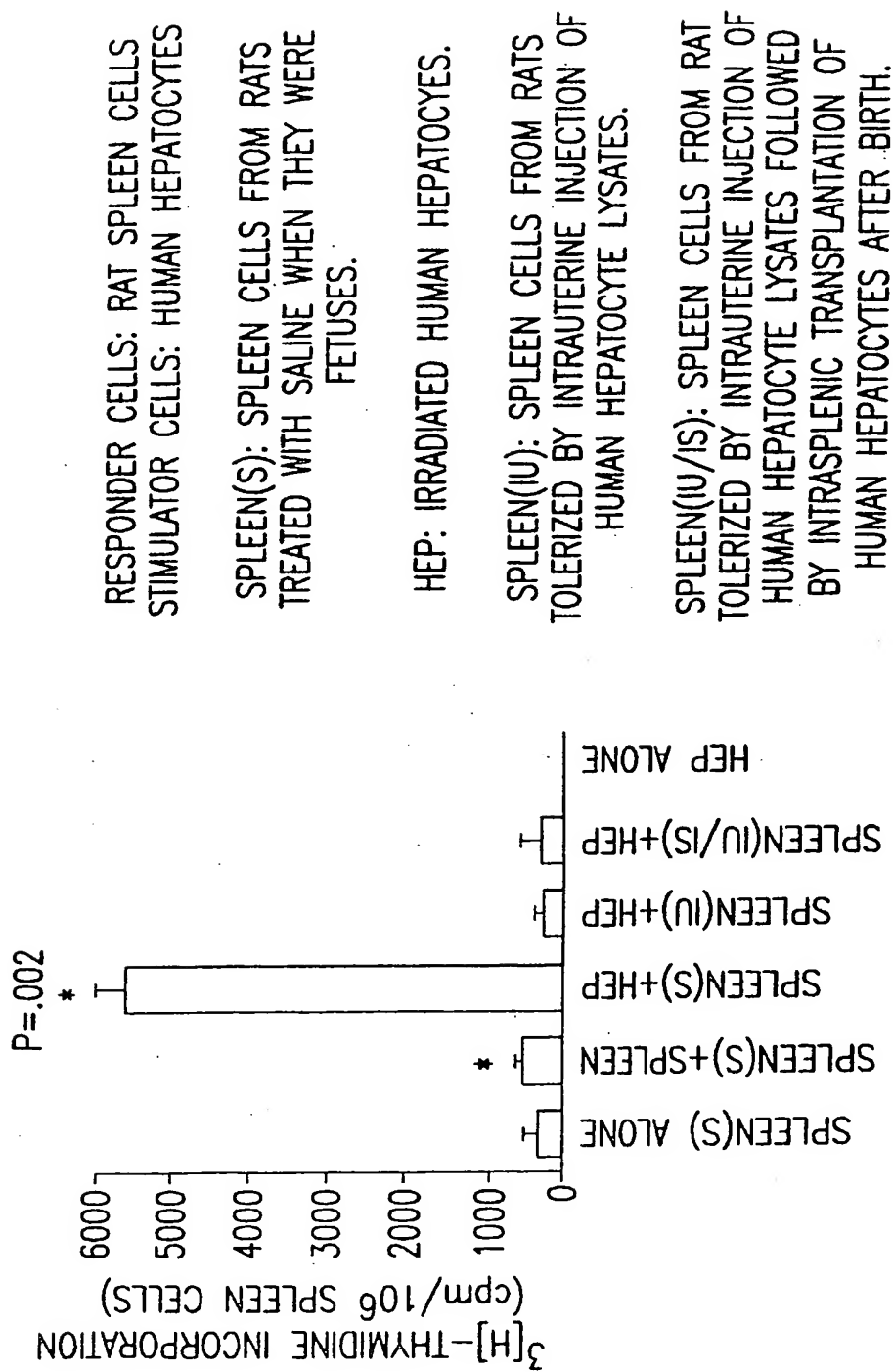


FIG.1

2/19

1 2 3 4 5

252 253

FIG.2

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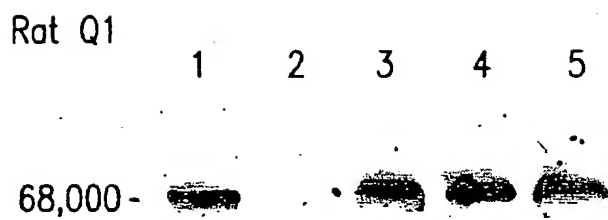


FIG.3

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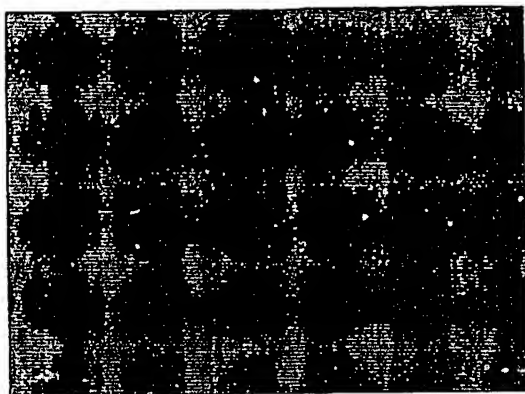


FIG. 4A



FIG. 4B

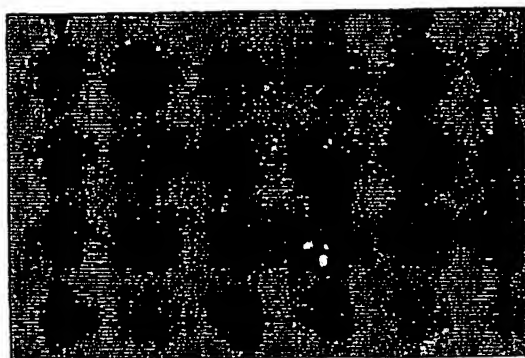


FIG. 4C



FIG. 4D



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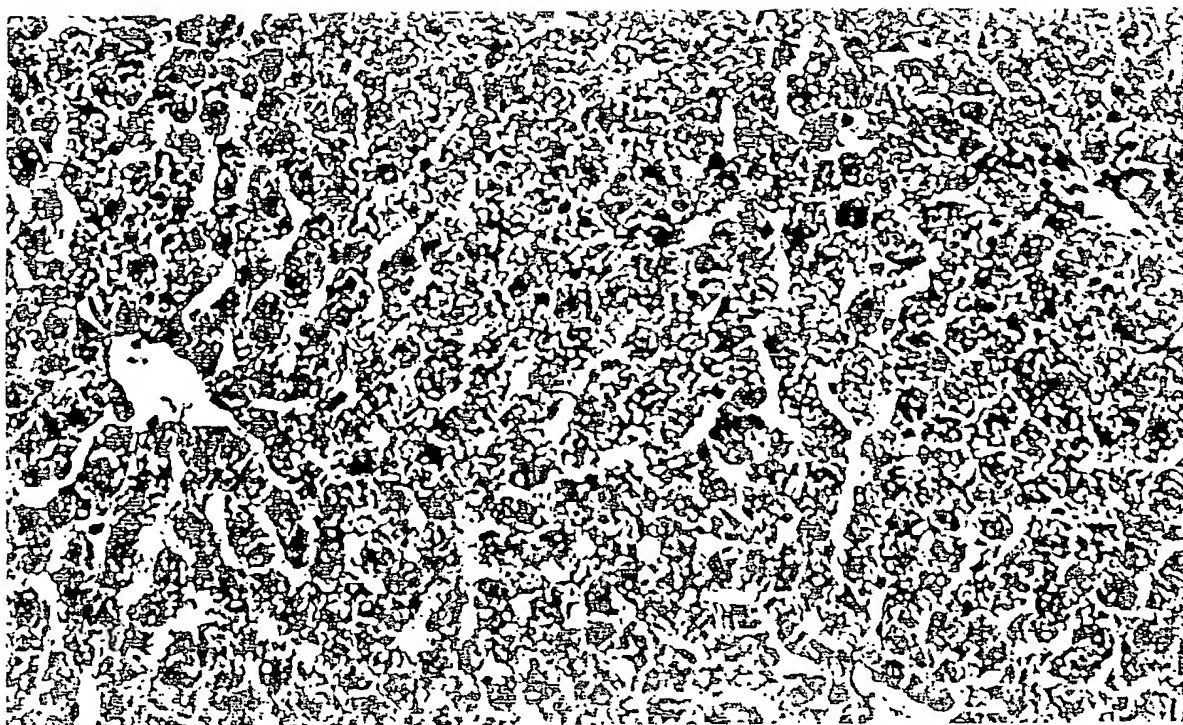


FIG.5

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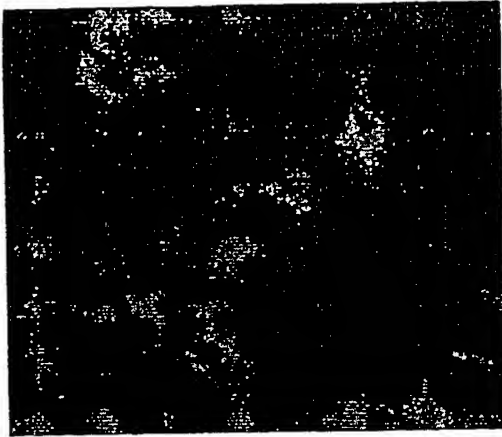


FIG. 6A

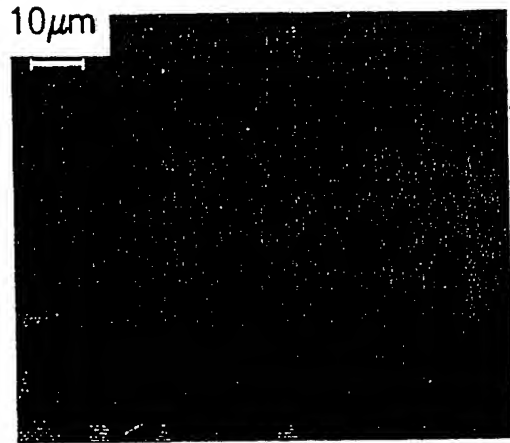


FIG. 6B

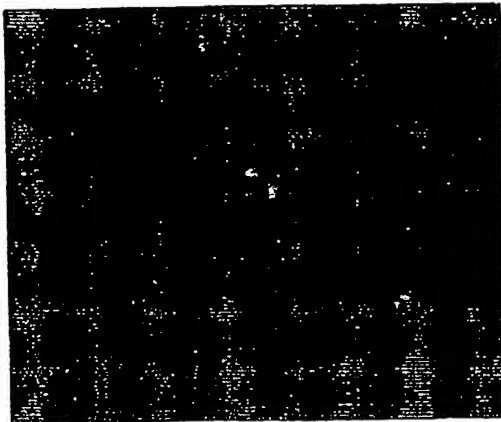


FIG. 6C

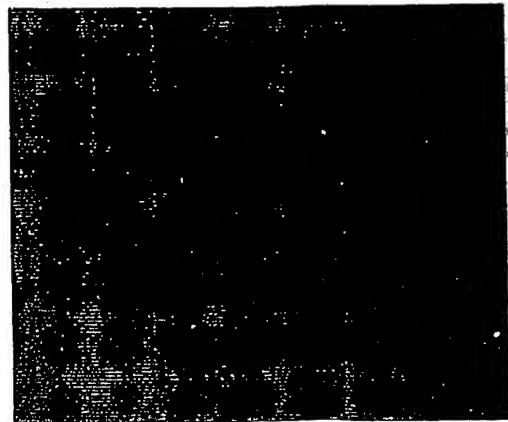
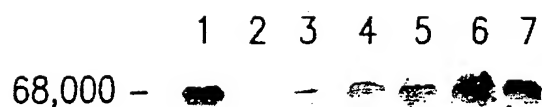


FIG. 6D

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- 1: 10 ng standard human albumin
- 2: 10 ng standard rat albumin
- 3: 2 days
- 4: 2 weeks
- 5: 3 weeks
- 6: 5 weeks
- 7: 6 weeks

FIG.7

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Time course of human albumin  
Anti Human Albumin

Anti Hepatitis B Surface Antigen

1 week

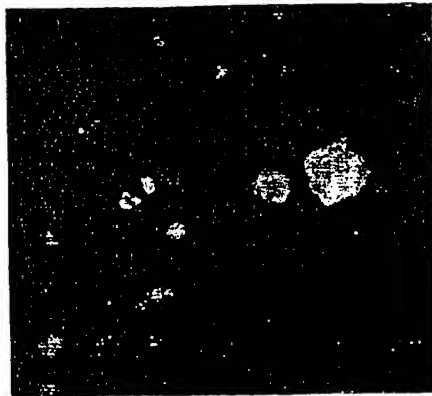


FIG. 8A



FIG. 8B

6 weeks

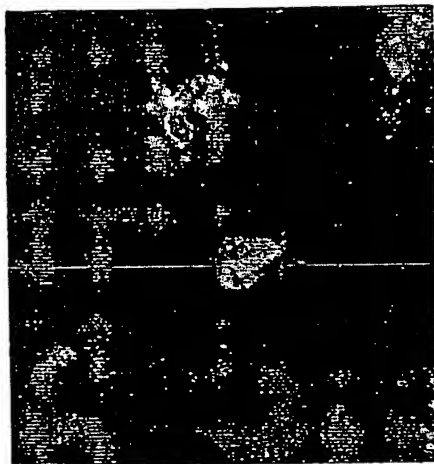


FIG. 8C

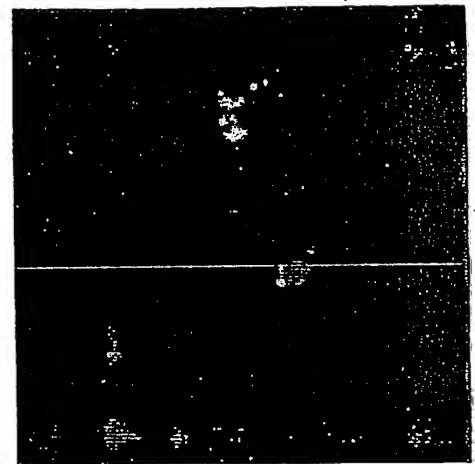


FIG. 8D

14 weeks

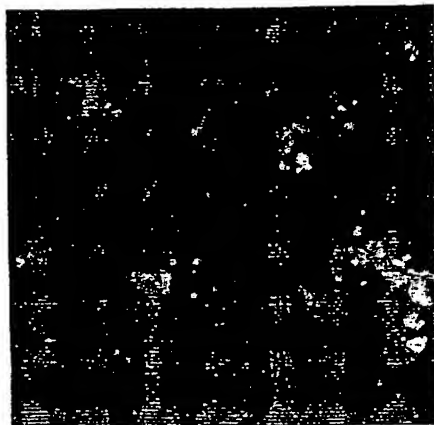


FIG. 8E

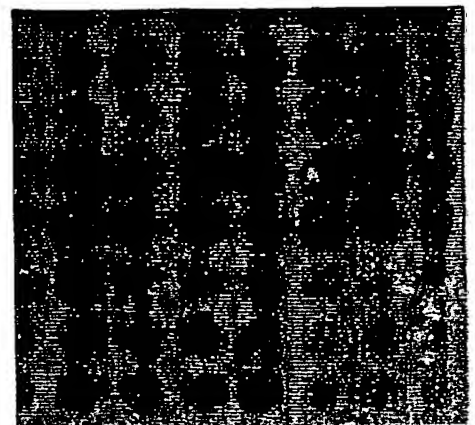


FIG. 8F

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Anti Human Albumin

Anti Hepatitis B Surface Antigen

Rat CA2  
Hepatocytes  
Plus HBV



FIG.9A



FIG.9B

Rat CA3  
Hepatocytes  
alone

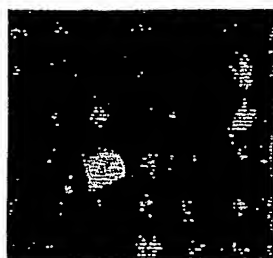


FIG.9C

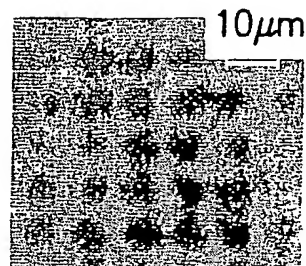


FIG.9D

Rat CA5  
HBV alone



FIG.9E

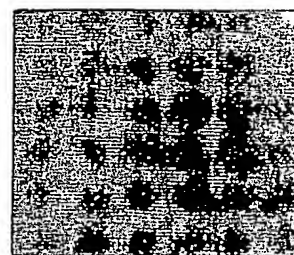


FIG.9F

Rat CA2  
minus primary Ab

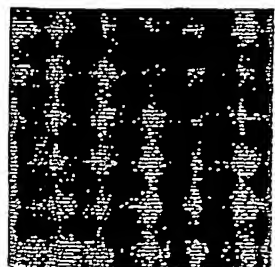


FIG.9G



FIG.9H

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## RT-PCR Human Albumin mRNA

- 1: 1000 bp ladder
- 2: Rat RNA
- 3: Human RNA
- 4: HepG2.2.15 RNA
- 5: Rat CA1 - Human hepatocytes + HBV
- 6: Rat CA2 - Human hepatocytes + HBV
- 7: Rat CA3 - Human hepatocytes
- 8: Rat CA4 - Human hepatocytes
- 9: Rat CA5 - HBV
- 10: Rat CA6 - HBV
- 11: Rat CA7 - Saline

FIG. 10

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## RT-PCR Human Albumin RNA

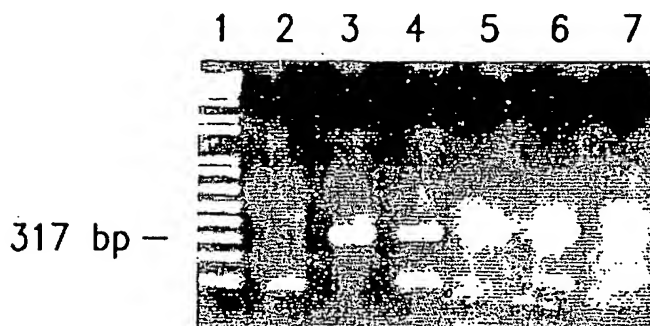
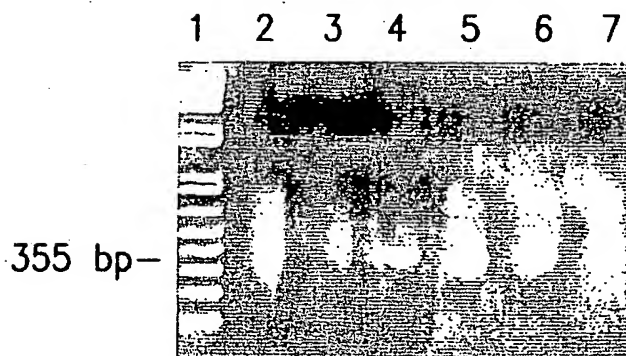


FIG. 11A

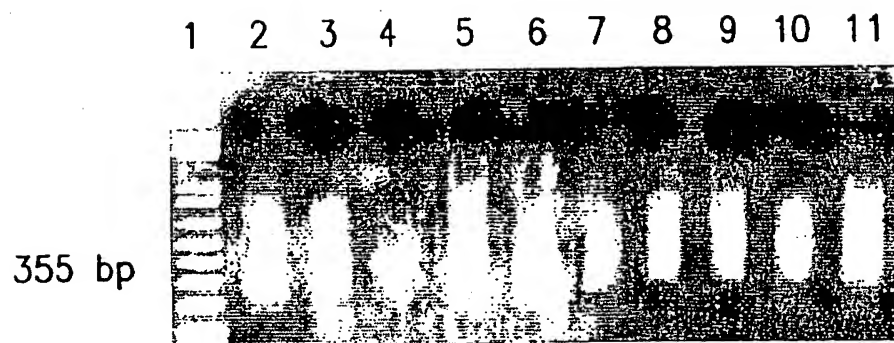
## RT-PCR HBV RNA



- 1: 1 kbp ladder
- 2: Rat liver RNA
- 3: Human liver RNA
- 4: HepG2.2.15 RNA
- 5: Rat CA2 - Human hepatocytes + HBV,  
1 week post
- 6: Rat CA2 - 6 weeks post
- 7: Rat CA2 - 14 weeks post

FIG. 11B

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- 1: 1000 bp ladder
- 2: Rat RNA
- 3: Human RNA
- 4: HepG2.2.15 RNA
- 5: Rat CA1 - Human hepatocytes + HBV
- 6: Rat CA2 - Human hepatocytes + HBV
- 7: Rat CA3 - Human hepatocytes
- 8: Rat CA4 - Human hepatocytes
- 9: Rat CA5 - HBV
- 10: Rat CA6 - HBV
- 11: Rat CA7 - Saline

FIG.12

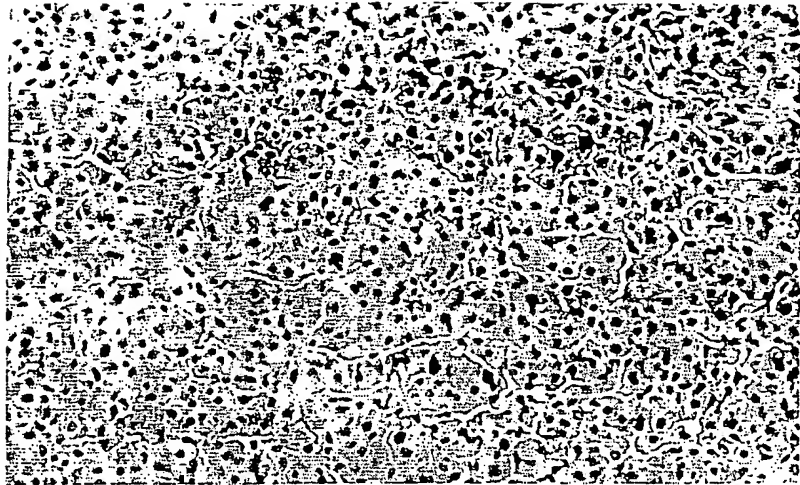


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Hepatocytes  
plus HBV

1 week

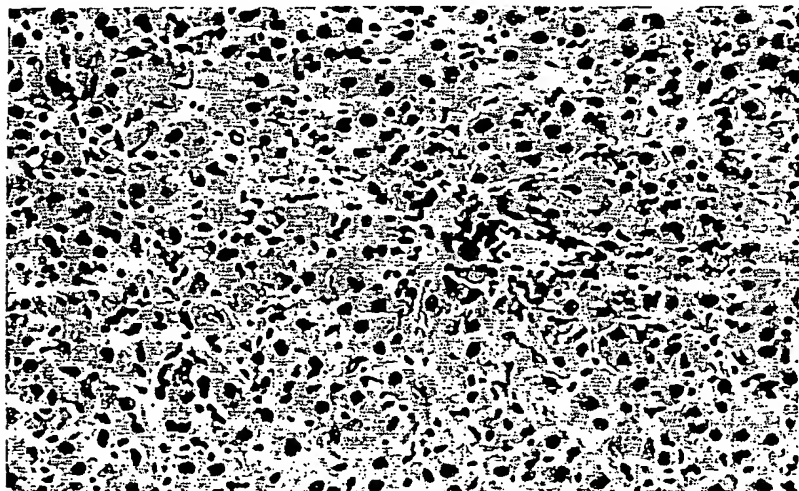
FIG.13A



Hepatocytes  
plus HBV

6 weeks

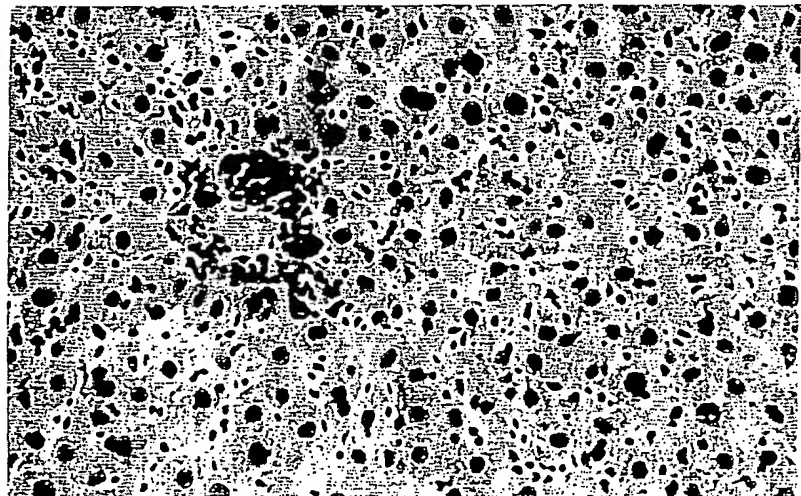
FIG.13B



Hepatocytes  
plus HBV

14 weeks

FIG.13C

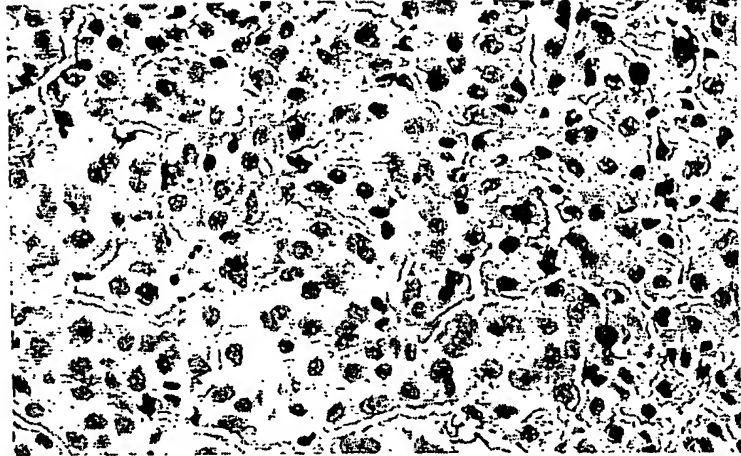


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Hepatocytes  
plus HBV

1 week

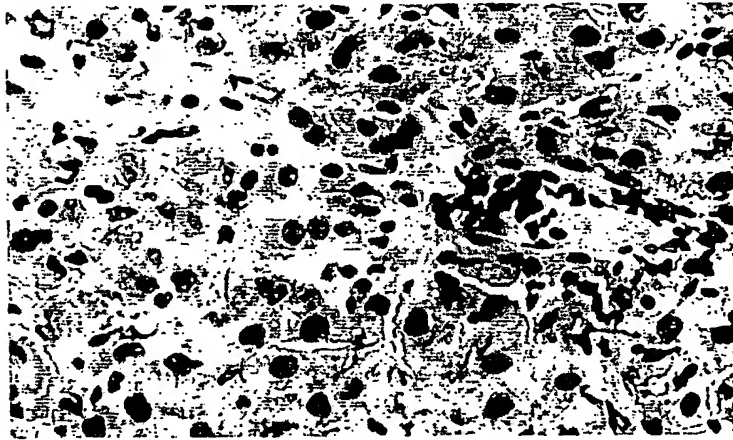
FIG.14A



Hepatocytes  
plus HBV

6 weeks

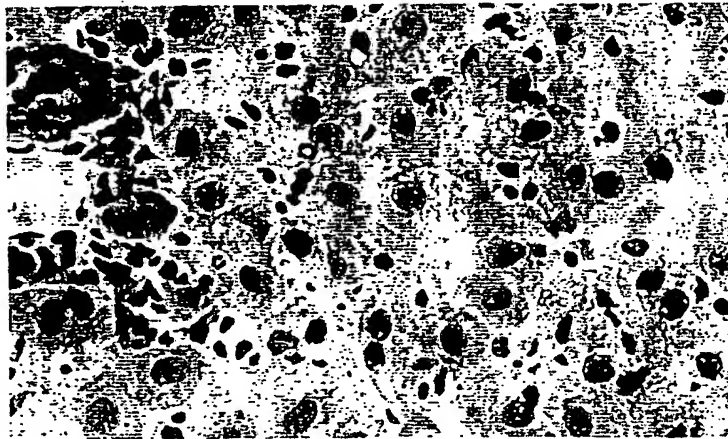
FIG.14B



Hepatocytes  
plus HBV

14 weeks

FIG.14C



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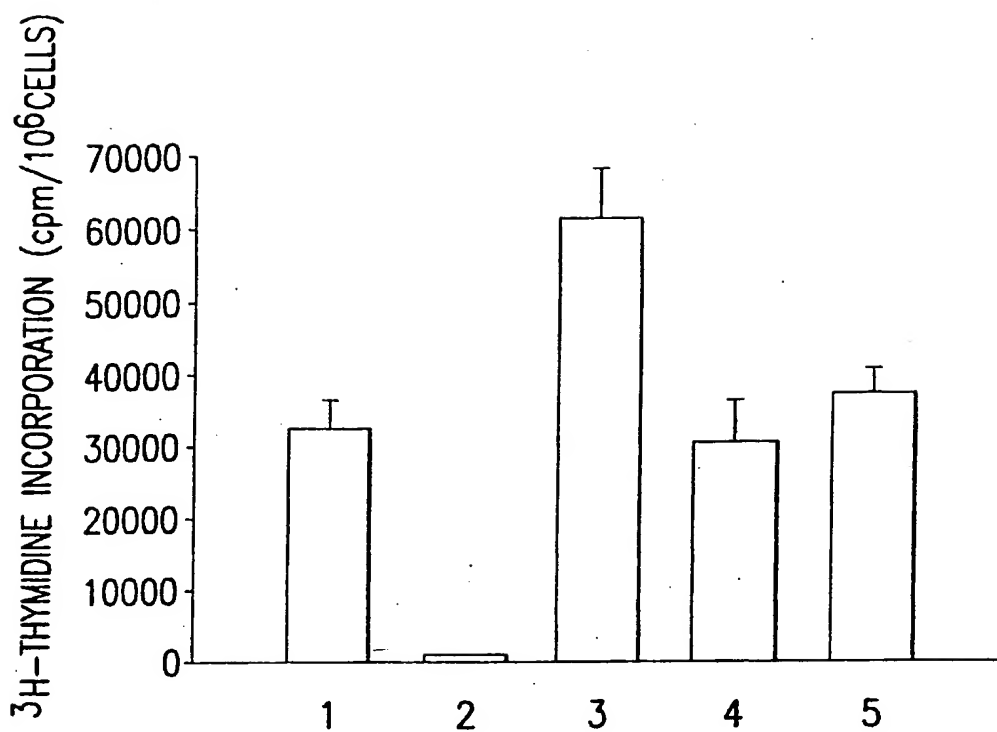


FIG. 15

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FIG. 16A

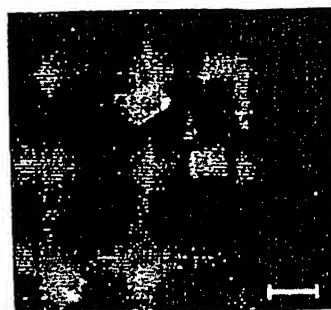


FIG. 16B

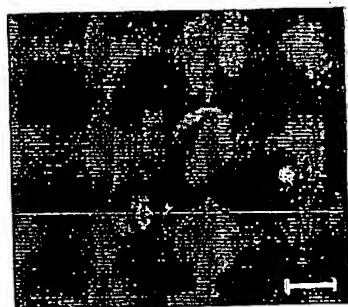


FIG. 16C



FIG. 16D

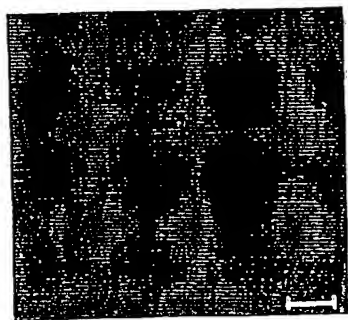


FIG. 16E



FIG. 16F

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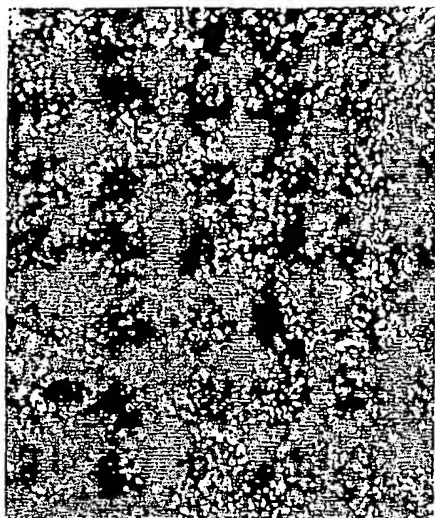


FIG. 17A

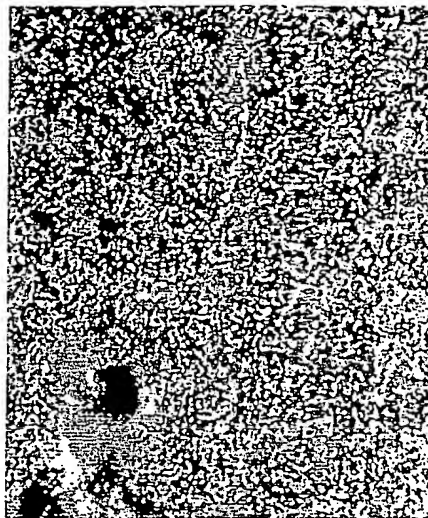


FIG. 17B

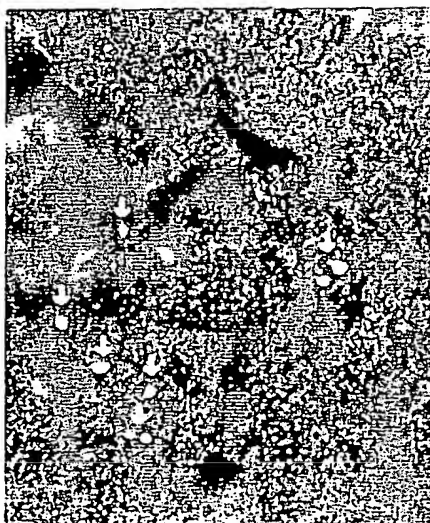


FIG. 17C

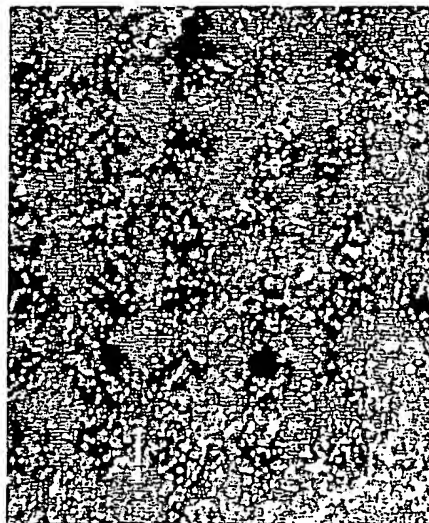


FIG. 17D

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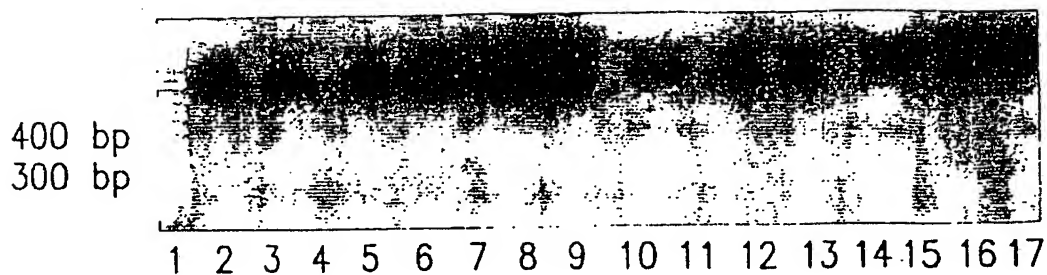


FIG.18A

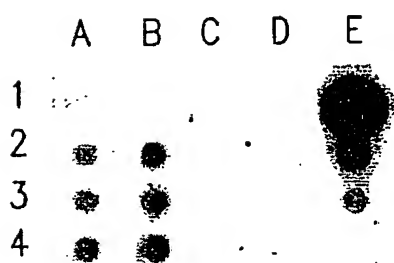


FIG.18B

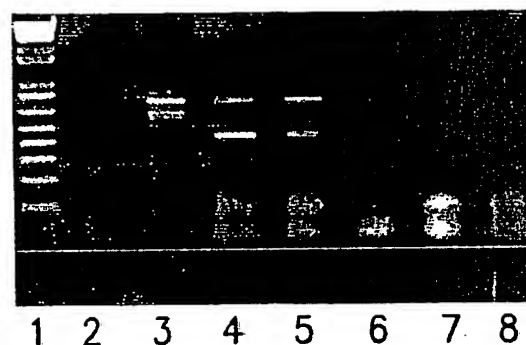


FIG.19A

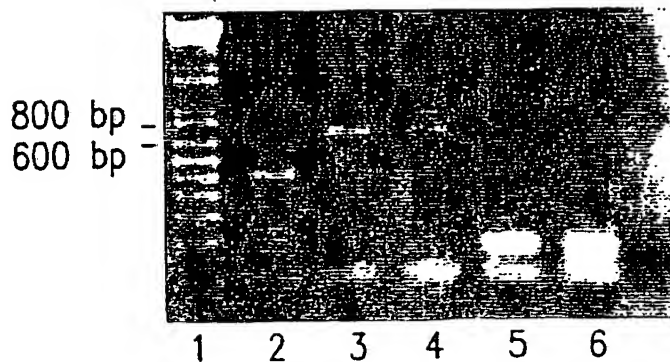


FIG.19B

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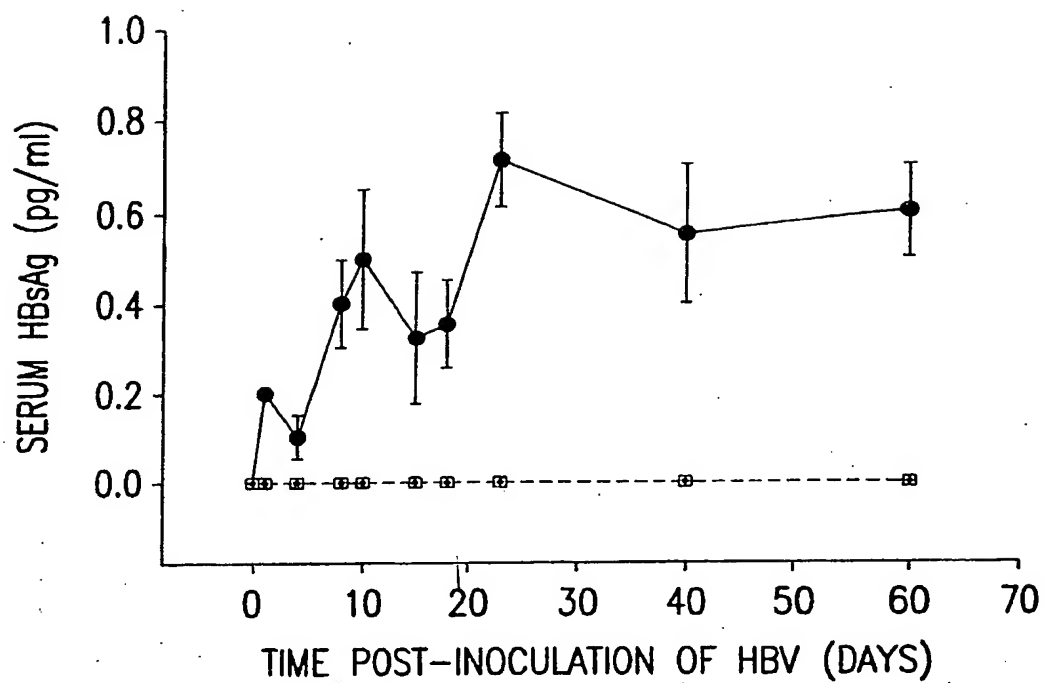


FIG.20

## SEQUENCE LISTING

<110> The University of Connecticut  
Wu, George Y.  
Wu, Catherine H.

<120> PROPAGATION OF HUMAN HEPATOCYTES IN  
NON-HUMAN ANIMALS

<130> 32407 PCT

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/05713

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01K 67/027, 67/033; A01N 63/00, 65/00; C12N 15/00, 15/09, 15/63

US CL : 424/93.1, 93.2, 93.21; 435/320.1; 800/9, 18

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1, 93.2, 93.21; 435/320.1; 800/9, 18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CAPLUS, WEST2.0

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| Y         | SU et al. Selective killing of AFP-positive hepatocellular carcinoma cells by adeno-associated virus transfer of the herpes simplex virus thymidine kinase gene. Human Gene Therapy. 01 March 1996, Vol. 7, No. 4, pages 463-470, see abstract.                            | 1, 5-9, 12, 18, 19    |
| Y         | Database CAPLUS, Accession No. 1990:569781, HECKEL et al. Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. Cell. 1990, Vol. 62, No. 3, pages 447-456, see abstract.   | 1-4, 9, 12, 13, 17    |
| X, P      | Database MEDLINE, Accession No. 2000080866, BROWN et al. A long-term hepatitis B viremia model generated by transplanting nontumorigenic immortalized human hepatocytes in Rag-2-deficient mice. Hepatology. 31 January 2000, Vol. 31, No. 1, pages 173-181, see abstract. | 24, 26, 34, 36        |
| Y, P      |  | 25, 27-33, 35, 37-42  |

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

|   |  |
|---|--|
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| *A* document defining the general state of the art which is not considered to be of particular relevance  | *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone   |
| *B* earlier document published on or after the international filing date  | *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
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| *O* document referring to an oral disclosure, use, exhibition or other means  |  |
| *P* document published prior to the international filing date but later than the priority date claimed  |  |

Date of the actual completion of the international search

07 MAY 2000

Date of mailing of the international search report

23 MAY 2000

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks

Box PCT

Washington, D.C. 20231

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/05713

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| X         | SACCI et al. Mouse model for exoerythrocytic stages of <i>Plasmodium falciparum</i> malaria parasite. Proceedings of the National Academy of Sciences USA. May 1992, Vol. 89, pages 3701-3705, see abstract. | 24, 26                |
| X, E      | US 6,034,297 A (J. VIERLING) 07 March 2000 (07.03.2000), see entire document, especially abstract.   | 24, 25                |
| Y         | WO 96/39810 A1 (SANDOZ LTD.) 19 December 1996 (19.12.1996), see entire document, especially pages 7 and 11.  | 1, 9, 20-22, 24-33    |